# RNATIONAL SEARCH REPORT

Inter• ɔnel Applicetion No
PCl/EP 97/04438

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C12N15/12 A01N63/02 A01H5/00

According to International Patent Classification(IPC) oi to both national classificetion and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed oy classification symbols)
IPC 6 C12N A01N A01H C07K

Documentation seerched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international seerch (name of data base and, where preclical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | ZHANG, Z., ET AL.: "Gene transfer for enhancing plant disease-resistance to bacterial pathogens" BIOTECHABS DATABASE, AN 96-00905, XP002048440 see abstract & HORTSCIENCE, vol. 30, no. 4, 1995, page 788 | 1,13 |
| Y | | 2-4,6,7, 10-12, 14-16 |

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are fisted in annex.

* Special categories of cited documents :

"A" document delining the generel state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the inferneticnal filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publicaticndate of enother citation or other special reeson (as epecified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published pnor to the international filing date but later then the priority date claimed

"T" later document published alter the international filing date oi priority date and not in conflict with the application but cited to understand the principle oi theory underlying the invention

"X" document of particular relevence: the claimed invention cannot be considered noval or cannot be considered to involve en inventive step when the document is taken alone

"Y" document of particuler relavance: the claimed invention cannot be considered to involve an inventive step when the document ia combined with one or more other such documents, such combination being obvious to e person skilled in the art.

"&" document member of the same patent family

1

| Date of the actual completion of theinternetional search | Date of mailing of the international search report |
|---|---|
| 5 December 1997 | 29/12/1997 |
| Name and mailing address of the ISA<br>European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax (+31-70) 340-3016 | Authorized officer<br>Maddox, A |

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | BELLAMY, W., ET AL.: "Identification of the bactericidal domain of lactoferrin" BIOCHIM. BIOPHYS. ACTA, vol. 1121, 1992, pages 130-136, XP002049260 see the whole document | 2-4,6,7, 10-12, 14-16 |
| X | ZHANG, ZHANYUAN 'PH.D.! ET AL: "DEVELOPMENT OF TRANSGENIC PLANTS WITH NON-PLANT ANTIBACTERIAL PROTEIN GENES FOR RESISTANCE TO BACTERIAL PATHOGENS ( LACTOFERRIN, NICOTIANA TABACUM, AGROBACTERIUM TUMEFACIENS)" DISSERTATION ABSTRACTS DATABASE AN 97:3204 & DISSERTATION ABSTRACTS INTERNATIONAL, (1996) VOL. 57, NO. 7B, P. 4124. ORDER NO.: AAR9637086. 185 PAGES., XP002048439 see the whole document | 1,13 |
| P,X | WO 96 37094 A (KOREA INST SCIENCE TECHNOLOGY ;LIU JANG RYOL (KR); LEE KYUNG KWANG) 28 November 1996 see the whole document | 1 |
| A | MITRA, A., ET AL.: "Expression of a human lactoferrin cDNA in tobacco cells produces antibacterial proteins(s)" PLANT PHYSIOLOGY, vol. 106, 1994, pages 977-981, XP002048441 see the whole document | 1-16 |
| A | YAMAUCHI, K., ET AL.: "Antibacterial activity of lactoferrin and a pepsin-derived lactoferrin peptide fragment" INFECTION AND IMMUNITY, vol. 61, no. 2, February 1993, pages 719-728, XP002049261 see the whole document | 1-16 |
| A | EP 0 474 506 A (MORINAGA MILK INDUSTRY CO LTD) 11 March 1992 see the whole document | 1-16 |
| A | DATABASE WPI Section Ch, Week 9551 Derwent Publications Ltd., London, GB; Class B04, AN 95-399338 XP002049263 & JP 07 274 970 A (MORINAGA MILK IND CO LTD) , 24 October 1995 see abstract | 1-16 |

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No |
|---|---|---|
| A | JAYNES, J.M., ET AL.: "Expression of a cecropin 8 lytic peptide analog in transgenic tobacco confers enhanced resistance to the bacterial wilt caused by Pseudomonas solanacearum" PLANT SCIENCE, vol. 89, 1993, pages 43-53, XP002049262 see the whole document | 1-16 |
| A | WO 90 11770 A (CALGENE INC) 18 October 1990 see the whole document | 1-16 |
| A | WO 89 04371 A (UNIV LOUISIANA STATE) 18 May 1989 see the whole document | 1-16 |
| A | WO 95 18859 A (CIBA GEIGY AG ;UNIV RESEARCH CORP (US); ROBERTS WALOEN K (US); SEL) 13 July 1995 see page 41, line 1 - line 5 | 2 |
| A | TERRAS F R G ET AL: "SMALL CYSTEINE-RICH ANTIFUNGAL PROTEINS FROM RAOISH: THEIR ROLE IN HOST OEFENSE" PLANT CELL, vol. 7, no. 5, May 1995, pages 573-588, XP002029836 see the whole document | 1-16 |

1

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 97/04438

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 9637094 A | 28-11-96 | NONE | |
| EP 0474506 A | 11-03-92 | JP 5092994 A | 16-04-93 |
| | | AU 645342 B | 13-01-94 |
| | | AU 8370491 A | 12-03-92 |
| | | CA 2050786 A | 08-03-92 |
| | | US 5304633 A | 19-04-94 |
| WO 9011770 A | 18-10-90 | CA 2030779 A | 12-10-90 |
| | | EP 0425616 A | 08-05-91 |
| WO 8904371 A | 18-05-89 | AU 2802989 A | 01-06-89 |
| | | CA 1321157 A | 10-08-93 |
| | | EP 0675960 A | 11-10-95 |
| | | US 5597945 A | 28-01-97 |
| WO 9518859 A | 13-07-95 | US 5521153 A | 28-05-96 |
| | | AU 1678995 A | 01-08-95 |
| | | EP 0738324 A | 23-10-96 |
| | | US 5559034 A | 24-09-96 |

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US 91/00887

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 N 15/82, C 12 N 5/10, A 01 H 5/00

## II. FIELDS SEARCHED

Minimum Documentation Searched [7]

| Classification System | Classification Symbols |
|---|---|
| IPC[5] | C 12 N, A 01 H |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

## III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

| Category * | Citation of Document, [11] with indication, where appropriate, of the relevant passages [12] | Relevant to Claim No. [13] |
|---|---|---|
| X | EP, A, 0295959 (PLANT CELL RESEARCH INST.) 21 December 1988 see page 5, column 8, lines 52-61; page 9, column 15, line 20 - column 16, line 20 -- | 1,11-13, 16,17,19, 20 |
| A | WO, A, 8402913 (MONSANTO) 2 August 1984 see pages 41-44 -- | 2-10,19, 20 |
| A | WO, A, 8700865 (CALGENE, INC.) 12 February 1987 see page 4, lines 16-17; page 9, line 32 - page 10, line 6 -- | 2-10,19, 20 |
| A | The EMBO Journal, vol. 7, no. 2, 1988, IRL Press Ltd, (Oxford, GB), Z.-L. Chen et al.: "A DNA sequence element that confers seed-specific enhancement to a constitutive promoter", pages 297-302 see the whole article -- | 1 |

./.

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 5th July 1991 | 30. 08. 91 |
| **International Searching Authority** | **Signature of Authorized Officer** |
| EUROPEAN PATENT OFFICE | M. PEIS M. Peis |

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category * | Citation of Document, ** with indication, where appropriate, of the relevant passages | Relevant to Claim No. |
|---|---|---|
| A | WO, A, 9001551 (ROGERS)<br>22 February 1990<br>see page 13, lines 1,2<br>-- | 2-10,19,20 |
| A | Plant Molecular Biology, vol. 13, November 1989, Kluwer Academic Publishers, (BE),<br>S.B. Altenbach et al.: "Enhancement of the methionine content of seed proteins by the expression of a chimeric gene encoding a methionine-rich protein in transgenic plants", pages 513-522<br>see the whole article<br>-- | 1,11-13,16, 17,19,20 |
| A | Journal of Cellular Biochemistry, suppl. 12C, 1988, Alan R. Liss, Inc., (New York, US),<br>see abstract L300<br>S.B. Altenbach et al.: "Processing of a Brazil nut sulfur-rich seed protein in transgenic plants", page 177<br>-- | 1,11-13,16, 17,19,20 |
| A | EP, A, 0319353 (PGS)<br>7 June 1989<br>see the whole document<br>cited in the application<br>---------- | 2-10,19,20 |

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. US 9100887
SA 46073

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 26/08/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| EP-A- 0295959 | 21-12-88 | AU-A- 1810088 | 22-12-88 |
| | | JP-A- 1091787 | 11-04-89 |
| WO-A- 8402913 | 02-08-84 | EP-A,B 0131623 | 23-01-85 |
| | | JP-T- 60500796 | 30-05-85 |
| WO-A- 8700865 | 12-02-87 | US-A- 4956282 | 11-09-90 |
| | | AU-B- 603063 | 08-11-90 |
| | | AU-A- 6196686 | 05-03-87 |
| | | EP-A- 0233915 | 02-09-87 |
| | | JP-T- 63500425 | 18-02-88 |
| WO-A- 9001551 | 22-02-90 | AU-A- 4037289 | 05-03-90 |
| | | EP-A- 0428572 | 29-05-91 |
| EP-A- 0319353 | 07-06-89 | AU-A- 2811889 | 23-05-89 |
| | | WO-A- 8903887 | 05-05-89 |
| | | EP-A- 0318341 | 31-05-89 |
| | | JP-T- 2501802 | 21-06-90 |

# PCT

## REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* RM/X89356/PC

**Box No. I TITLE OF INVENTION**

Synthetic polynucleotide coding for human lactoferrin, vectors, cells and transgenic plants containing it

**Box No. II APPLICANT**

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

PLANTECHNO Srl
Via Staffolo 60
26040 Vicomoscano CR
ITALY

☐ This person is also inventor.

Telephone No.

Facsimile No.

Teleprinter No.

State *(that is, country)* of nationality: ITALY

State *(that is, country)* of residence: ITALY

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

**Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)**

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

FOGHER Corrado
Via Ticino 32
26041 Casalmaggiore CR
ITALY

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is,country)* of nationality: ITALY

State *(that is, country)* of residence: ITALY

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on a continuation sheet.

**Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE**

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name: for a legal entity, full official designation. The address must include postal code and name of country.)*

Mario LEONE
Società Italiana Brevetti S.p.A.
Piazza di Pietra 39
00186 Roma
ITALY

Telephone No.
+39 06 695441

Facsimile No.
+39 06 69544830

Teleprinter No.

☐ Adress for correspondence: Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

## Box No. V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

- [x] AP **ARIPO Patent:** GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SL Sierra Leone, SZ Swaziland, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- [x] EA **Eurasian Patent:** AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- [x] EP **European Patent:** AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- [x] OA **OAPI Patent:** BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*

- [x] AE United Arab Emirates
- [x] AL Albania
- [x] AM Armenia
- [x] AT Austria
- [x] AU Australia
- [x] AZ Azerbaijan
- [x] BA Bosnia and Herzegovina
- [x] BB Barbados
- [x] BG Bulgaria
- [x] BR Brazil
- [x] BY Belarus
- [x] CA Canada
- [x] CH and LI Switzerland and Liechtenstein
- [x] CN China
- [x] CU Cuba
- [x] CZ Czech Republic
- [x] DE Germany
- [x] DK Denmark
- [x] EE Estonia
- [x] ES Spain
- [x] FI Finland
- [x] GB United Kingdom
- [x] GD Grenada
- [x] GE Georgia
- [x] GH Ghana
- [x] GM Gambia
- [x] HR Croatia
- [x] HU Hungary
- [x] ID Indonesia
- [x] IL Israel
- [x] IN India
- [x] IS Iceland
- [x] JP Japan
- [x] KE Kenya
- [x] KG Kyrgyzstan
- [x] KP Democratic People's Republic of Korea
- [x] KR Republic of Korea
- [x] KZ Kazakhstan
- [x] LC Saint Lucia
- [x] LK Sri Lanka
- [x] LR Liberia
- [x] LS Lesotho
- [x] LT Lithuania
- [x] LU Luxembourg
- [x] LV Latvia
- [x] MD Republic of Moldova
- [x] MG Madagascar
- [x] MK The former Yugoslav Republic of Macedonia
- [x] MN Mongolia
- [x] MW Malawi
- [x] MX Mexico
- [x] NO Norway
- [x] NZ New Zealand
- [x] PL Poland
- [x] PT Portugal
- [x] RO Romania
- [x] RU Russian Federation
- [x] SD Sudan
- [x] SE Sweden
- [x] SG Singapore
- [x] SI Slovenia
- [x] SK Slovakia
- [x] SL Sierra Leone
- [x] TJ Tajikistan
- [x] TM Turkmenistan
- [x] TR Turkey
- [x] TT Trinidad and Tobago
- [x] UA Ukraine
- [x] UG Uganda
- [x] US United States of America
- [x] UZ Uzbekistan
- [x] VN Viet Nam
- [x] YU Yugoslavia
- [x] ZA South Africa
- [x] ZW Zimbabwe

Check-boxes reserved for designating States which have become party to the PCT after issuance of this sheet:

- [ ] .....
- [ ] .....

**Precautionary Designation Statement:** In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)*

**Supplemental Box** *If the Supplemental Box is not used, this sheet should not be included in the request.*

*1. If, in any of the Boxes, the space is insufficient to furnish all the information: in such case, write "Continuation of Box No. ..." [indicate the number of the Box] and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient, in particular:*

*(i) if more than two persons are involved as applicants and/or inventors and no "continuation sheet" is available: in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below;*

*(ii) if, in Box No. II or in any of the sub-boxes of Box No. III, the indication "the States indicated in the Supplemental Box" is checked: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;*

*(iii) if, in Box No. II or in any of the sub-boxes of Box No. III, the inventor or the inventor/applicant is not inventor for the purposes of all designated States or for the purposes of the United States of America: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the inventor(s) and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;*

*(iv) if, in addition to the agent(s) indicated in Box No. IV, there are further agents: in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;*

*(v) if, in Box No. V, the name of any State (or OAPI) is accompanied by the indication "patent of addition," or "certificate of addition," or if, in Box No. V, the name of the United States of America is accompanied by an indication "continuation" or "continuation-in-part": in such case, write "Continuation of Box No. V" and the name of each State involved (or OAPI), and after the name of each such State (or OAPI), the number of the parent title or parent application and the date of grant of the parent title or filing of the parent application;*

*(vi) if, in Box No. VI, there are more than three earlier applications whose priority is claimed: in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI;*

*(vii) if, in Box No. VI, the earlier application is an ARIPO application: in such case, write "Continuation of Box No. VI", specify the number of the item corresponding to that earlier application and indicate at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed.*

*2. If, with regard to the precautionary designation statement contained in Box No. V, the applicant wishes to exclude any State(s) from the scope of that statement: in such case, write "Designation(s) excluded from precautionary designation statement" and indicate the name or two-letter code of each State so excluded.*

*3. If the applicant claims, in respect of any designated Office, the benefits of provisions of the national law concerning non-prejudicial disclosures or exceptions to lack of novelty: in such case, write "Statement concerning non-prejudicial disclosures or exceptions to lack of novelty" and furnish that statement below.*

Continuation of Box No. IV

ADORNO Silvano, AIMI Luciano, BARDINI Marco Luigi, BAZZICHELLI Alfredo, BORRINI Stefano, CONCONE Emanuele, DE BENEDETTI Fabrizio, DI CERBO Mario, IACOBELLI Daniele, MOSCONE BENVENUTI Francesca, PELLEGRI Alberto, PIZZOLI Antonio Maria, PIZZOLI Pasquale, STEINFL Alessandro, STRINI Giorgio, TONON Gilberto

Società Italiana Brevetti S.p.A.
Piazza di Pietra 39
00186 Roma
Italy

**Box No. VI PRIORITY CLAIM** ☐ Further priority claims are indicated in the Supplemental Box.

| Filing date of earlier application (day/month/year) | Number of earlier application | Where earlier application is: national application: country | regional application:* regional Office | international application: receiving Office |
|---|---|---|---|---|
| item (1) 17 July 1998 | RM98A000478 | ITALY | | |
| item (2) | | | | |
| item (3) | | | | |

☐ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s): _____

* *Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.*

**Box No. VII INTERNATIONAL SEARCHING AUTHORITY**

**Choice of International Searching Authority (ISA)** *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):*
ISA /

**Request to use results of earlier search; reference to that search** *(if an earlier search has been carried out by or requested from the International Searching Authority):*
Date *(day/month/year)* Number Country *(or regional Office)*

**Box No. VIII CHECK LIST; LANGUAGE OF FILING**

This international application contains the following number of sheets:

| | |
|---|---|
| request | : 4 |
| description (excluding sequence listing part) | : 57 |
| claims | : 4 |
| abstract | : 1 |
| drawings | : 13 Provv. |
| sequence listing part of description | : 16 |
| **Total number of sheets** | : 95 |

This international application is accompanied by the item(s) marked below:

1. ☐ fee calculation sheet
2. ☐ separate signed power of attorney
3. ☐ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ priority document(s) identified in Box No. VI as item(s):
6. ☐ translation of international application into (language):
7. ☐ separate indications concerning deposited microorganism or other biological material
8. ☒ nucleotide and/or amino acid sequence listing in computer readable form
9. ☐ other *(specify)*:

**Figure of the drawings which should accompany the abstract:** 16

**Language of filing of the international application:** ITALIAN

**Box No. IX SIGNATURE OF APPLICANT OR AGENT**

*Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).*

Mario LEONE

**For receiving Office use only**

| | |
|---|---|
| 1. Date of actual receipt of the purported international application: | 2. Drawings: ☐ received: ☐ not received: |
| 3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application: | |
| 4. Date of timely receipt of the required corrections under PCT Article 11(2): | |
| 5. International Searching Authority (if two or more are competent): ISA / | 6. ☐ Transmittal of search copy delayed until search fee is paid. |

**For International Bureau use only**

Date of receipt of the record copy by the International Bureau:

*The demand must be filed directly with the competent International Preliminary Examining Authority or, if two or more Authorities are competent, with the one chosen by the applicant. The full name or two-letter code of that Authority may be indicated by the applicant on the line below:*

IPEA/ _____

PCT: REC'D 16 JAN 2001 **CHAPTER II**

# DEMAND

under Article 31 of the Patent Cooperation Treaty:
The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty and hereby elects all eligible States (except where otherwise indicated).

For International Preliminary Examining Authority use only

| Identification of IPEA | Date of receipt of DEMAND |
|---|---|
| | |

| **Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION** | | Applicant's or agent's file reference RM/X89356/PC-EBR |
|---|---|---|
| International application No. PCT/IT99/00226 | International filing date *(day/month/year)* 19 July 1999 | (Earliest) Priority date *(day/month/year)* 17 July 1998 |

Title of invention

A synthetic polynucleotide coding for human lactoferrin, vectors, cells and transgenic plants containing it

**Box No. II APPLICANT(S)**

| Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)* | Telephone No.: |
|---|---|
| PLANTECHNO Srl<br>Via Staffolo 60<br>26040 Vicomoscano CR<br>ITALY | Facsimile No.: |
| | Teleprinter No.: |

| State *(that is, country)* of nationality: | State *(that is, country)* of residence: |
|---|---|
| ITALY | ITALY |

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

FOGHER Corrado
Via Ticino 32
26041 Casalmaggiore CR
ITALY

| State *(that is, country)* of nationality: | State *(that is, country)* of residence: |
|---|---|
| ITALY | ITALY |

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

| State *(that is, country)* of nationality: | State *(that is, country)* of residence: |
|---|---|
| | |

☐ Further applicants are indicated on a continuation sheet.

International application No.
PCT/IT99/00226

**Box No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE**

The following person is [X] agent [ ] common representative

and [X] has been appointed earlier and represents the applicant(s) also for international preliminary examination.

[ ] is hereby appointed and any earlier appointment of (an) agent(s)/common representative is hereby revoked.

[ ] is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

BAZZICHELLI Alfredo
Società Italiana Brevetti S.p.A.
Piazza di Pietra 39
00186 Roma
ITALY

Telephone No.: +39 06 695441

Facsimile No.: +39 06 69544830

Teleprinter No.:

[ ] Address for correspondence: Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special addr ess to which correspondence should be sent.

**Box No. IV BASIS FOR INTERNATIONAL PRELIMINARY EXAMINATION**

Statement concerning amendments: *

1. The applicant wishes the international preliminary examination to start on the basis of:

[X] the international application as originally filed

the description [X] as originally filed
[ ] as amended under Article 34

the claims [X] as originally filed
[ ] as amended under Article 19 (together with any accompanying statement)
[ ] as amended under Article 34

the drawings [X] as originally filed
[ ] as amended under Article 34

2. [ ] The applicant wishes any amendment to the claims under Article 19 to be considered as reversed.

3. [ ] The applicant wishes the start of the international preliminary examination to be postponed until the expiration of 20 months from the priority date unless the International Preliminary Examining Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69.1(d)). *(This check-box may be marked only where the time limit under Article 19 has not yet expired.)*

* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments of the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

Language for the purposes of international preliminary examination: ENGLISH

[ ] which is the language in which the international application was filed.
[X] which is the language of a translation furnished for the purposes of international search.
[ ] which is the language of publication of the international application.
[ ] which is the language of the translation (to be) furnished for the purposes of international preliminary examination.

**Box No. V ELECTION OF STATES**

The applicant hereby elects all eligible States *(that is, all States which have been designated and which are bound by Chapter II of the PCT)*

excluding the following States which the applicant wishes not to elect:

International application No.
PCT/IT99/00226

**Box No. VI CHECK LIST**

| The demand is accompanied by the following elements, in the language referred to in Box No. IV, for the purposes of international preliminary examination: | | | For International Preliminary Examining Authority use only: received | not received |
|---|---|---|---|---|
| 1. translation of international application | : | sheets | ☐ | ☐ |
| 2. amendments under Article 34 | : | sheets | ☐ | ☐ |
| 3. copy (or, where required, translation) of amendments under Article 19 | : | sheets | ☐ | ☐ |
| 4. copy (or, where required, translation) of statement under Article 19 | : | sheets | ☐ | ☐ |
| 5. letter | : | sheets | ☐ | ☐ |
| 6. other *(specify)* | : | sheets | ☐ | ☐ |

The demand is also accompanied by the item(s) marked below:

1. [x] fee calculation sheet
2. [ ] separate signed power of attorney
3. [ ] copy of general power of attorney; reference number, if any:
4. [ ] statement explaining lack of signature
5. [ ] nucleotide and or amino acid sequence listing in computer readable form
6. [ ] other *(specify)*:

**Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE**

*Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand).*

A Bazzichelli

Alfredo BAZZICHELLI

**For International Preliminary Examining Authority use only**

1. Date of actual receipt of DEMAND:
2. Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b):
3. ☐ The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5, below, does not apply. ☐ The applicant has been informed accordingly.
4. ☐ The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80.5.
5. ☐ Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82.

**For International Bureau use only**

Demand received from IPEA on:

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

BAZZICHELLI, A.
Società Italiana Brevetti S.p.A.
Piazza di Pietra 39
00186 Roma
ITALIE

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

| Date of mailing *(day/month/year)* | 08.12.2000 |
|---|---|

| Applicant's or agent's file reference | IMPORTANT NOTIFICATION |
|---|---|
| RM/X89356/PC-EBR | |

| International application No. | International filing date *(day/month/year)* | Priority date *(day/month/year)* |
|---|---|---|
| PCT/IT99/00226 | 19/07/1999 | 17/07/1998 |

Applicant
PLANTECHNO SRL et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

| Name and mailing address of the IPEA/ | Authorized officer |
|---|---|
| European Patent Office<br>D-80298 Munich<br>Tel. +49 89 2399 - 0 Tx: 523656 epmu d<br>Fax: +49 89 2399 - 4465 | Emslander, S<br><br>Tel.+49 89 2399-8718 |

Form PCT/IPEA/416 (July 1992)

[redacted]TENT COOPERATION TRE[redacted]Y

# PCT

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

| Applicant's or agent's file reference | FOR FURTHER ACTION | See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416) |
|---|---|---|
| RM/X89356/PC-EBR | | |
| International application No. | International filing date *(day/month/year)* | Priority date *(day/month/year)* |
| PCT/IT99/00226 | 19/07/1999 | 17/07/1998 |
| International Patent Classification (IPC) or national classification and IPC | | |
| C12N15/12 | | |
| Applicant | | |
| PLANTECHNO SRL *et al.* | | |

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 5 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☒ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

| Date of submission of the demand | Date of completion of this report |
|---|---|
| 03/02/2000 | 06.12.2000 |
| Name and mailing address of the international preliminary examining authority:<br>European Patent Office<br>D-80298 Munich<br>Tel. +49 89 2399 - 0 Tx: 523656 epmu d<br>Fax: +49 89 2399 - 4465 | Authorized officer<br>Hecki, K<br>Telephone No. +49 89 2399 8430 |

Form PCT/IPEA/409 (cover sheet) (January 1994)

# INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IT99/00226

## I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
**Description, pages:**

| | | | | |
|---|---|---|---|---|
| 1-54 | as originally filed | | | |

**Claims, No.:**

| | | | | |
|---|---|---|---|---|
| 1-49 | as received on | 01/11/2000 | with letter of | 01/11/2000 |

**Drawings, sheets:**

| | |
|---|---|
| 1-13 | as originally filed |

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any ***nucleotide and/or amino acid sequence*** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   - ☐ the description, pages:
   - ☐ the claims, Nos.:

**INTERNATIONAL PRELIMINARY EXAMINATION REPORT**

International application No. PCT/IT99/00226

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

*(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)*

6. Additional observations, if necessary:

**IV. Lack of unity of invention**

1. In response to the invitation to restrict or pay additional fees the applicant has:

☒ restricted the claims.

☒ paid additional fees.

☐ paid additional fees under protest.

☐ neither restricted nor paid additional fees.

2. ☐ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

☐ complied with.

☒ not complied with for the following reasons:
**see separete sheet**

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

☐ all parts.

☒ the parts relating to claims Nos. 1-34,41-49part.

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

| | | | |
|---|---|---|---|
| Novelty (N) | Yes: | Claims | 2,3,6-13,24-34,41-49part |
| | No: | Claims | 1,4,5,14-23, |
| Inventive step (IS) | Yes: | Claims | |
| | No: | Claims | 2,3,6-13,24-34,41-49part |

| | | | |
|---|---|---|---|
| Industrial applicability (IA) | Yes: | Claims | 1-34,41-49part |
| | No: | Claims | |

2. Citations and explanations
**see seperete sheet**

**INTERNATIONAL PRELIMINARY EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/IT99/00226

Re Item IV

**Reasoned statement under Art.34(3) and Rules 13 and 68 PCT with regard to unity of invention; citations and explanations supporting such statement.**

1. The international search report has been drawn up in respect of the entire international application but the International Preliminary Examining Authority is of the opinion that the application does not comply with the requirements of unity of invention as set forth in the PCT regulations (Article 34(3), Rules 13 and 68 PCT):

2. The separate inventions/groups of invention are:

(i) a polynucleotide coding for human lactoferrin, the lactoferrin polypeptide, expression vectors for lactoferrin, the use thereof in plant transformation, plants and plant cells expressing human lactoferrin (claims 1, 3, 42 all complete and claims 1-28)

(ii) a plant expression cassette allowing the tissue specific expression of a gene of interest comprising the promoter of the gene coding for the protein basic globulin 7S, the use thereof in plant transformation, plants and plant cells expressing the gene of interest (claims 29-34 totally, claims 41-49 partially)

(iii) a plant expression cassette allowing the tissue specific expression of a gene of interest comprising the promoter of the gene coding for the beta-conclycinin protein, the use thereof in plant transformation, plants and plant cells expressing the gene of interest (claims 35-40 totally, claims 41-49 partially)

3. They are not so linked as to form a single general inventive concept for the following reasons:

The common concept linking together subject-matter (i) on the one hand and subject-matter (ii) and (iii) on the other hand is - at the best - the expression of a gene of interest in transgenic plants. This is well known in the art (see for example the prior art cited in the description). In addition, the use of tissue specific regulation elements is also part of the prior art (see e.g. WO91/13993 or WO 98/06860, cited in the ISR). Accordingly there is also no common inventive

concept linking together subject-matter (ii) and (iii).

The Applicant payed one additional fee under protest and restricted the claims to the subject-matter (i) and (ii) as referred to under item 2. Therefore, this s.m. is the subject of this IPER.

**Re Item V**

**Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement.**

1. Reference is made to the following documents:

D1: SALMON V. ET AL.: 'Production of human lactoferrin in transgenic tobacco plants' PROTEIN EXPRESSION AND PURIFICATION, vol. 13, 1998, pages 127-135, XP000863470

D2: MITRA A ET AL: 'EXPRESSION OF A HUMAN LACTOFERRIN CDNA IN TOBACCO CELLS PRODUCES ANTIBACTERIAL PROTEIN(S)' PLANT PHYSIOLOGY,US,AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, ROCKVILLE, MD, vol. 106, no. 3, 1994, page 977-981 XP002048441 ISSN: 0032-0889

D3: WARD P P ET AL: 'A SYSTEM FOR PRODUCTION OF COMMERCIAL QUANTITIES OF HUMAN LACTOFERRIN: A BROAD SPECTRUM NATURAL ANTIBIOTIC' BIO/TECHNOLOGY,US,NATURE PUBLISHING CO. NEW YORK, vol. 13, 1995, page 498-503 XP002048442 ISSN: 0733- 222X

D4: ZHANG ET AL: 'DEVELOPMENT OF TRANSGENIC PLANTS WITH NON-PLANT ANTIBACTERIAL PROTEIN GENES FOR RESISTANCE TO BACTERIAL PATHOGENS ( LACTOFERRIN, NICOTIANA TABACUM, AGROBACTERIUM TUMEFACIENS)' DIALOG DISSERTATION ABSTRACTS,XX,XX,1996, XP002048439

D5: WO 96 37094 A (KOREA INST SCIENCE TECHNOLOGY ;LIU JANG RYOL (KR); LEE KYUNG KWANG) 28 November 1996 (1996-11-28)

D6: WO 91 13993 A (UPJOHN CO) 19 September 1991 (1991-09-19)

D7: WO 98 06860 A (CIBA GEIGY AG ;VERNOOIJ BARNARDUS THEODORUS M (NL); CHANDLER DANIE) 19 February 1998 (1998-02-19)

D8: WATANABE Y ET AL: 'Nucleotide sequence of the basic 7S globulin gene

from soybean.' PLANT PHYSIOLOGY, (1994 JUL) 105 (3) 1019-20. , XP002125177

D9: FR-A-2 762 850 (BIOCEM) 6 November 1998 (1998-11-06)

2. Novelty (Art.33(2) PCT):

D1 - D5 and D7 disclose polynucleotides encoding human lactoferrin (hLF), vectors for the (tissue specific) expression of a gene of interest, transformation process, transgenic plants and the polypeptide obtained by the expression of the polynucleotide, (see D1, abstract and Discussion; D2, abstract and materials and methods; D3, abstract; D4, abstract; D5, page 2, line 31 to page 5, line 34; D7, end of page 2 to page end of page 12, in particular page 6, lines 20-25, page 22 to page 23).

Hence, the subject-matter of claims 1, 4, 5, 14-23 is not novel.

3. Inventiveness (Art.33(3) PCT):

3.1 D6 is considered closest prior art. D6 discloses seed specific plant expression cassettes which comprise a gene of interest operatively linked to regulation elements allowing the tissue specific expression of said gene, optionally linked to a leader sequence, the regulation sequence being among others beta-conglycinine promoter, the gene of interest bovine growth hormone, transgenic plants (see D6, page 2, line 10 to page 3, line 29, page 5, line 11 to page 9, line 27, examples 1-11).

Following the disclosure of any of documents D1-D5 is obvious that the human lactoferrin gene may equally be the gene of interest in the teaching of D6.

Therefore, the claims referring to the expression of human lactoferrin and to expression cassettes in combination with the beta-conglycinine derived promoter and leader sequences is rendered obvious.

3.2 The same applies to the use of the cell type specific sequences of the basic globulin 7S polypeptide which is rendered obvious by D6 (see D6, page 1, lines

22-31). It is also within the scope of the skilled person to select and adapt appropriate sequences therefore (see to D8 referring to the basic 7S globulin gene from soybean).

3.3 The use of hLF expressing plants for the production of hLF and for pharmaceutic and nutritional needs is rendered obvious by the prior art documents D1 (see D1, end of discussion), D3 (see D3, abstract), and D6 (see D6, summary).

Accordingly, the subject-matter of claims 2, 3, 6-13, 24-28, 29-34, 41-49 lacks an inventive step.

4. No English translation of the priority document pertaining to the present application has been available at the time of establishing this communication. Therefore, this communication has been based on the assumption that the relevant parts of the claims enjoy the priority claimed.

Should it later turn out that this is not the case D9 cited as P/X-document in the International search report could become relevant to the subject-matter of the claims.

PATENT COOPERATION TREATY



From the INTERNATIONAL BUREAU

# PCT

## NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

To:
LEONE, Mario
Società Italiana Brevetti S.p.A.
Piazza di Pietra, 39
I-00186 Roma
ITALIE



IMPORTANT NOTICE

| Date of mailing (day/month/year) | |
|---|---|
| 27 January 2000 (27.01.00) | |

| Applicant's or agent's file reference | |
|---|---|
| RM/X89356/PC | |

| International application No. | International filing date (day/month/year) | Priority date (day/month/year) |
|---|---|---|
| PCT/IT99/00226 | 19 July 1999 (19.07.99) | 17 July 1998 (17.07.98) |

Applicant
PLANTECHNO SRL et al

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AU,CN,EP,IL,JP,KP,KR,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   AE,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,CA,CH,CU,CZ,DE,DK,EA,EE,ES,FI,GB,GD,GE,GH,GM,HR,HU,ID,IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MD,MG,MK,MN,MW,MX,NO,NZ,OA,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,UA,UG,UZ,VN,YU,ZA,ZW
   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   27 January 2000 (27.01.00) under No. WO 00/04146

### REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, a **demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

### REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national** phase, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer
J. Zahra

Telephone No. (41-22) 338.83.38

[redacted] Continuation of Form PCT/IB/308 [redacted]

## NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

| Date of mailing (day/month/year) | IMPORTANT NOTICE |
|---|---|
| 27 January 2000 (27.01.00) | |
| **Applicant's or agent's file reference** | **International application No.** |
| RM/X89356/PC | PCT/IT99/00226 |

The applicant is hereby notified that, at the time of establishment of this Notice, the time limit under Rule 46.1 for making amendments under Article 19 has not yet expired and the International Bureau had received neither such amendments nor a declaration that the applicant does not wish to make amendments.

M-11

# PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

## PCT

### NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

| Date of mailing (day/month/year) | |
|---|---|
| 02 March 2000 (02.03.00) | |
| **International application No.** | **Applicant's or agent's file reference** |
| PCT/IT99/00226 | RM/X89356/PC |
| **International filing date (day/month/year)** | **Priority date (day/month/year)** |
| 19 July 1999 (19.07.99) | 17 July 1998 (17.07.98) |
| **Applicant** | |
| FOGHER, Corrado | |

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

03 February 2000 (03.02.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

| The International Bureau of WIPO<br>34, chemin des Colombettes<br>1211 Geneva 20, Switzerland | Authorized officer<br>S. Mafla |
|---|---|
| Facsimile No.: (41-22) 740.14.35 | Telephone No.: (41-22) 338.83.38 |

Form PCT/IB/331 (July 1992) 3142424

TENT COOPERATION TREATY

# PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

| Applicant's or agent's file reference | FOR FURTHER ACTION | see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below. |
|---|---|---|
| RM/X89356/PC | | |
| International application No. | International filing date *(day/month/year)* | (Earliest) Priority Date *(day/month/year)* |
| PCT/IT 99/00226 | 19/07/1999 | 17/07/1998 |
| Applicant | | |
| PLANTECHNO SRL et al. | | |

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Seerch Report consists of e total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the intemationel search wes carried out on the basis of the intemational application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search wes cerried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to eny **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [X] contained in the intemational application in written form.

   [X] filed together with the intemationel application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the International application as filed has been fumished.

   [X] the statement thet the information recorded in computer readable form is identical to the written sequence listing has been fumished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of Invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is epproved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text Is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority es it appeers in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 16

   [X] as suggested by the applicent.

   [ ] None of the figures.

   [ ] because the epplicant failed to suggest e figure.

   [ ] because this figure better characterizes the invention.

# INTERNATIONAL SEARCH REPORT

[redacted]national Application No
PCT/IT 99/00226

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C12N15/82 C12N5/10 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | SALMON V. ET AL.: "Production of human lactoferrin in transgenic tobacco plants" PROTEIN EXPRESSION AND PURIFICATION, vol. 13, 1998, pages 127-135, XP000863470 | 1,3, 37-42 |
| Y | see the whole document, esp. discussion | 2,4,5,8, 9,19-21, 24, 28-30, 35,36 |
| | --- -/-- | |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 23 December 1999 | 11/01/2000 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL – 2280 HV Rijswijk<br>Tel. (+31–70) 340–2040, Tx. 31 651 epo nl,<br>Fax: (+31–70) 340–3016 | Kania, T |

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | MITRA A ET AL: "EXPRESSION OF A HUMAN LACTOFERRIN CDNA IN TOBACCO CELLS PRODUCES ANTIBACTERIAL PROTEIN(S)" PLANT PHYSIOLOGY,US,AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, ROCKVILLE, MD, vol. 106, no. 3, 1994, page 977-981 XP0D2048441 ISSN: 0032-0889 the whole document | 3,42 |
| X | WARD P P ET AL: "A SYSTEM FOR PRODUCTION OF COMMERCIAL QUANTITIES OF HUMAN LACTOFERRIN: A BROAO SPECTRUM NATURAL ANTIBIOTIC" BIO/TECHNOLOGY,US,NATURE PUBLISHING CO. NEW YORK, vol. 13, 1995, page 498-503 XP002048442 ISSN: 0733-222X the whole document | 3,42 |
| X | ZHANG ET AL: "DEVELOPMENT OF TRANSGENIC PLANTS WITH NON-PLANT ANTIBACTERIAL PROTEIN GENES FOR RESISTANCE TO BACTERIAL PATHOGENS ( LACTOFERRIN, NICOTIANA TABACUM, AGROBACTERIUM TUMEFACIENS)" OIALOG O1SSERTATION ABSTRACTS,XX,XX,1996, XP002D48439 abstract | 3,42 |
| X | WO 96 37D94 A (KOREA INST SCIENCE TECHNOLOGY ;LIU JANG RYOL (KR); LEE KYUNG KWANG) 28 November 1996 (1996-11-28) the whole document | 3,42 |
| X | WO 91 13993 A (UPJOHN CO) 19 September 1991 (1991-09-19) | 4,5, 8-11, 15-19, 24-28, 31-36 |
| Y | see the whole document, esp. examples 4 and 9 | 2,4,5,8, 9,19-21, 24, 28-30, 35,36 |
| A | WO 98 0686D A (CIBA GEIGY AG ;VERNOOIJ BARNARDUS THEODORUS M (NL); CHANOLER OANIE) 19 February 1998 (1998-02-19) see the whole document, esp. page 4, example 11 | 1-42 |

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication,where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | WATANABE Y ET AL: "Nucleotide sequence of the basic 7S globulin gene from soybean." PLANT PHYSIOLOGY, (1994 JUL) 105 (3) 1019-20. , XP002125177 the whole document | 6,7,23 |
| P,X | FR 2 762 850 A (BIOCEM) 6 November 1998 (1998-11-06) the whole document | 3-5,10, 11,13, 15-20, 25-29, 31-42 |

# INTERNATIONAL SEARCH REPORT

[redacted]nation on patent family members

[redacted]ternational Application No
[redacted]CT/IT 99/00226

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 9637094 A | 28-11-1996 | EP 0831688 A | 01-04-1998 |
| | | JP 10506540 T | 30-06-1998 |
| | | US 5914448 A | 22-06-1999 |
| WO 9113993 A | 19-09-1991 | AU 7583691 A | 10-10-1991 |
| | | EP 0519011 A | 23-12-1992 |
| WO 9806860 A | 19-02-1998 | AU 4205297 A | 06-03-1998 |
| | | CN 1228123 A | 08-09-1999 |
| | | EP 0918873 A | 02-06-1999 |
| | | PL 331563 A | 19-07-1999 |
| FR 2762850 A | 06-11-1998 | AU 7659298 A | 27-11-1998 |
| | | WO 9850543 A | 12-11-1998 |

# A SYNTHETIC POLYNUCLEOTIDE CODING FOR HUMAN LACTOFERRIN, VECTORS, CELLS AND TRANSGENIC PLANTS CONTAINING IT

## DESCRIPTION

### Field of the invention

The present invention relates to the field of the vegetable biotechnologies, and in particular to the plants and vegetal cells transformation techniques and systems, to cells and transgenic plants thus generated, and to their use.

### State of the art

In recent years, due to the large variety of applications deriving from the utilization of the genetic engineering techniques in the biology of the vegetals, the use of genetically modified plants has gradually increased.

In particular, the development of techniques for the transformation of plants into organisms capable of producing proteins of commercial interest has acquired a remarkable practical importance.

In fact, the generation of recombinant plants containing a heterologous gene of interest, and their use in production processes on an industrial scale, allows to overcome a series of drawbacks characterizing the production systems presently in use, particularly those based on cell cultures.

Indeed, recombinant DNA technology allowed the generation of transgenic cells that are used in the production of heterologous proteins of interest. In particular, animal (and specifically mammal) cell cultures allow the production of proteins of interest, even extremely complex ones, in their native form, but the related process is extremely expensive, as hectoliters of cells are required for the production of an amount of proteins sufficient for a commercial (e.g., pharmacological or alimentary) use (Stowell et al., 1991).

An alternative in this direction is then provided by

the production, carried out in prokaryotic cell cultures, a cheaper process, meeting however a serious obstacle in the inability of those systems to effect the post transcriptional and post-translational modifications required for the expression of complex heterologous proteins, carried out only by the eukaryotic systems (Glick and Pasternak, 1994).

Therefore, a possible solution was pursued in the transformation of complex eukaryotic systems which could ensure at the same time the production of active proteins and the utilization of inexpensive processes. (Watson et al. 1992).

In particular, plants possess the required potential, and their capacity of functioning as bioreactors for the production of complex proteins (plants are highly efficient in this type of processes, ensuring a high degree of expression), in a cost-effective way (plant cultivation is relatively inexpensive) and with a high yield (each single harvest can yield high amounts of proteins) was proved (Fraley and Schell, 1991).

Further, very many plants satisfy the important requirement of the non-allergenicity needed for the production systems of recombinant proteins of pharmacological or alimentary interest. In fact, the organisms used in those productions must belong to the GRAS (Generally Recognized As Safe) organism category, i.e. organisms that having been used by man for a long time, are considered safe for man and for the ecosystem as well. Obviously, plants such as Leguminosae, cereals, tobacco, horticultural plants in general and fruit trees, satisfy this requirement in nature. Among leguminosae, Soya is a basic food in the diet of many populations mostly of the Third World Countries, but recently in European Countries as well. In fact, Soya-derived compounds constitute ingredients that are present in a vast number of food products, such as e.g., lecithin and

seed oil, while the seeds of this Leguminosa yield a flour that is employed in various food like soybean milk containing approximately the 8% thereof.

Therefore plants, while being the raw material from which the product can be extracted according to conventional processes, at the same time constitute an alternative to the conventional production. In fact, they can be used as functional foods, i.e. foods genetically modified so as to be enriched from a nutritional point of view, and in case assuming important properties as a natural drug.

Thus the step of the protein extraction from the plant has been eliminated, and the heterologous protein expression does not prelude to an extraction and purification thereof, steps that account for most of the production cost of a drug, but simply enriches a vegetable nutriment, which thus becomes a *nutriceutical*, i.e. a nutriment having a pharmaceutical value.

This is the reason for the research efforts aimed at generating genetically modified plants, optimized for the above-mentioned applications.

However, to date the evaluation of the heterologous expression capacity typical of various plant species was exclusively performed under laboratory conditions, or anyhow on relatively limited surfaces in hothouse.

For instance, expression in plants of enkephalin and human serum albumin, as well as of mice monoclonal antibodies was studied (Watson et al., 1992). More recently, always referring to proteins relevant from a pharmacological point of view, two additional human proteins of therapeutic importance, i.e. active interleukin-6 and C protein (a serum anticoagulant), were successfully expressed in tobacco. In all these cases the model plant on which the functionality of the prepared gene was tested is tobacco, whereas usually the plant finally selected for production is a leguminosa, whose seed content in storage proteins is high.

These experiments proved that often the expression levels of the heterologous protein in the vegetal bioreactors do not reach high enough levels to meet the commercial demands, and that anyhow it can be improved applying new information on the plant gene control. Specifically, it was demonstrated that the level of produced recombinant protein has to reach more than 1% of the total protein amount in order to become economically significant, a level obviously not achieved by the simple introduction of the heterologous gene, whose expression therefore needs to be maximized.

In relation to the vegetal cell biology, in order to maximize the level of the in plant expression, an action on various levels is necessary: increase of genic transcription, increase of transcript stability and of the translation process yield. Moreover, it is further necessary to fix the inserted gene and to minimize the risk of the occurrence of silencing or of genic inactivation. All these factors are crucial in assessing the in plant expression level of the heterologous gene, that, as afore stated, apart from some exceptions usually settles at rather low levels (Owen and Pen, 1996).

Among all these, the most crucial factor, together with the transcription level depending on the preferential presence of certain codons, would seem to be the stability of the recombinant protein in the heterologous host, as the likely probable cause of its easy elimination. The relative instability might be the consequence, on one hand of the inability of the translated heterologous protein to assume a stable structural conformation, on the other hand of the ultrastructural compartment in which it is directed after the translation, where the presence of proteases and of particular pH values determine its degradation.

Accordingly, it is therefore important both to provide the modification of the heterologous sequences in order to ensure the codon optimization and to carry out a

careful selection of the targeting sequences, capable of directing the translated protein into preferential ultrastructural compartments, as i.e. the seed storage tissue, capable of ensuring the stability of the product. Concerning the latter issue, the best strategy of action in this sense, is that of providing the maintenance of the endogenous signal sequences of the plant selected for the final production. In fact, the adoption of these sequences prevents the alteration of the cell internal balance, consequence of the unavoidable random accumulation of exogenous proteins that would take place in their absence. In this regard, the fact that the ultrastructural compartments have different characteristics in the cells of the various tissues has to be taken into account.

For instance, the heterologous proteins accumulated together with the storage proteins of the seeds in the transgenic plants, are more stable as compared with those of the vegetative organs (Owen and Pen, 1996). Probably, the reason of such different stability can be found in the different protease activities recorded in the vacuoles of the leaf meristem cells, with respect to those observed in the vacuoles of the seed. It is therefore understandable how research efforts are aimed at the generation of transgenic plants in which the heterologous protein be preferably expressed in the seed (Owen and Pen, 1996).

Furthermore, overall the seed constitutes the vegetal organ most used by man for its ease of conservation and, obviously, for its caloric and protein contribution. The seed consists of the plant embryo enveloped by storage tissues that provide energy and nitrogen during germination. Usually the storage tissue function is mainly effected by the endosperm, but in Leguminosae, as e.g. Soya, the cotyledons develop remarkably, and acquire this function.

The storage function for the nitrogen component is

carried out by particular proteins accumulated in the protein bodies, inside compartments in the endocellular membrane systems. The amount of proteins in the seed varies from about 10-15% in cereals to about 25-35% in Leguminosae, therefore seeds are an important protein source in the human diet (Shewry et al., 1995).

In order to exploit the expression system of the seed storage proteins, first of all it has to be considered that the storage proteins of all plants have some functional and physiological common characteristics: their synthesis is controlled during the seed ripening according to the nutritional needs, and they are stored in protein bodies. In particular, the Leguminosae storage proteins are divided in two classes: globulins and lectins. Globulins are the most widespread storage proteins, present not only in leguminosa but in monocotyledons and in other dicotyledons as well. The globulin class in turn is subdivided into two subclasses: legumins (11S hexameric proteins) and vicilins (7S trimeric proteins). Also β-conglycinine and basic 7S globulin, whose regulation elements were used to perform tissue-specific expression in seed (Shewry et al., 1995) belong to the vicilin subclass, but whereas the former was studied in detail, no detailed information is available on the basic globulin functioning.

β-conglycinine is a storage protein of the Soya seed (Glycine max.), consisting of three different subunits, α, α', β that interact non-covalently to form a trimer complex. The subunits are coded by a multigene family of 15 genes grouped in six nuclear DNA regions, whose expression is strictly regulated so as to be modulated during the plant life (Harada et at. 1989). Control is tissue-specific as well as stage-specific. In fact, the expression of each subunit is activated at high levels at the moment of embryo development, from the heart shaped phase until complete ripening, whereas it is repressed before the dormancy phase.

Moreover, expression occurs exclusively on specific plant zones, like cotyledons, according to an expression pattern that varies in the course of their ripening; at first it is activated on the outer cotyledon area, then a wave distribution from the outside to the inside is observed and lastly it turns out to be uniformly spread over the entire cotyledon tissue. However, during the heart shaped phase (18 days after pollination), the gene encoding the β-conglycinine also expresses itself in the embryonal axis, whereas it does not express in endosperm, tegmen and in already differentiated tissues (Perez-Grau and Goldberg, 1989). The same behavior also occurs in the seed of transgenic plants belonging to other families, as e.g., tobacco, proving the same control mechanism to be functional in solanaceae as well (Naito et al. 1988; Perez-Grau and Goldberg, 1989).

The regulation of subunits α/α' and β genic expression occur at a transcriptional as well as a post-transcriptional level (Harada et al., 1989). α' subunit, of 76 Kd, with a 2.5 Kb mRNA, is accumulated more precociously and in a larger amount as compared to β subunit. This behavior is due to the greater strength of the α' subunit due to the presence of an enhancer region, absent in β, and of a sequence stabilizing the α' transcript, it also absent in β (probably a 560 pb region in the first exon of the α' transcript) (Harada et al., 1989). This difference in expression has also been highlighted in transformed tobacco plants (Bray et al., 1987).

In contrast with the α' subunit, the expression level of the β subunit, is also influenced by abiotic stresses, methionine level in the ground and presence of ABA. The base elements involved in gene control at transcriptional level for the α' subunit are clustered in the 905 pb region at 5' of the transcription start site, region called URS (Upstream Regulatory Sequence). Inside this area specific sequences functioning as site-specific

enhancers have been detected. Among these, the legumin boxes (5'-CATGCAC.3' and 5'-CATGCAT-3'), elements that are found in many other genes encoding seed-specific proteins, in particular in legumes. A coordinated action of the two sequences determines a 10-fold increase of the seed gene expression level. The regulation by the above elements seems to be of a positive kind (Chamberland et al. 1992), however so far trans-elements specifically interacting in those sites have not yet been found. Site-specific expression also requires the coordinated action of elements operating in cis, not yet characterized, located in the region at 5' of the legumin boxes and at 3' of the promoter (Chamberland et al., 1992). Probably the region at 5' includes a negative control site, specific for a nuclear factor present only in non-seed tissues. This factor would determine gene expression in embryonal tissues only. The importance of CAAT and TATA sequences in the control of site-specificity has also been proved.

Four Soya nuclear factors that interact with specific sequences present in the α' and β URS of the gene have been identified as well. Two of those embryonal factors, SEF3 and SEF4, bind to sites inside the enhancer region (from -257 to -79 ). SEF3 binds to the middle of the sequence 5'-AACCCA.....AACCCA-3', present exclusively in the gene encoding the α'subunit. Accumulation and degradation of this protein (SEF3) is paralleled by accumulation and degradation of α' mRNA, supporting the hypothesis that SEF3 be involved in the control of α' expression. As compared to SEF3, SEF4 is present in a lesser amount, has many recognition sites (5'-A/GTTTTTA/G-3') in α', but mostly in β. The presence of this factor is correlated to the regulation of β expression (Lessard et al., 1991). Deletion and site-specific mutagenesis experiments have proved that the sole action of these factors does not affect the site-specificity nor the expression level, coordination with

the activity of the other regulatory elements being necessary. However, on the basis of the homology with light-induced proteins, these proteins are supposed to have a regulating role only under certain conditions (Fujiwara and Beachy, 1994).

Embryonal factors with a behavior similar to the SEF3, as verified in gel shift experiments, have been found in tobacco as well (Lessard et al., 1991). This and other data obtained with GUS activity assays under control of Soya α' promoter prove that the site- and stage-specific control mechanism is conserved (Lessard et al, 1991; Riggs et al., 1989). It has also been hypothesized that tobacco trans factors binding to 35SCaMV promoter may also interact with the legumin boxes (Katagiri et al., 1989). None of the afore mentioned factors appears to be directly responsible of the time regulation, and no NRS-like factor has been found possessing a negative control as in the case of bean (Bustos et al. 1991).

More recent studies, concerning some legumins and vicilins in Vicia Faba, contradict some generalizations on the regulation of the storage proteins expression in seeds (Wobus et al., 1995), showing that expression of genes of B4 and LeB4 Vicia Faba legumins is not limited to embryonal tissues, nor are they temporally restricted to the cell expansion phase in embryogenesis. Proteins are stored for short periods of time and then degraded in all embryonal tissues, suspensor and endosperm included, within well-defined developmental stages. This is so probably in order to allow an uninterrupted supply to the embryo of compounds having a high C and N content during all the growth and differentiation stages. Therefore, this data allow to hypothesize that the seed proteins expression be also controlled metabolically, and not merely at a developmental stages level. The possible relationship existing between storage proteins accumulation and carbohydrate metabolism (soluble glycid

level) is presently being investigated. Since all classes of seed storage proteins share a similar behavior in the different species, this data require a careful evaluation of the behavior, in terms of expression, also for the Soya proteins β-conglycinine and 7S basic globulin. Data resulting from the study, which the present invention is based on, clearly show the tissutal specificity of the expression of the structural portion of lactoferrin under control of both the promoters. Instead, the activation phase was at the present not investigated in detail as the sole capability of total seed accumulation was of interest. Specifically concerning the 7S basic globulin, it is a storage protein of the Soya seed, with a high methionine and cysteine content. Alike β-conglycinine, also 7S basic globulin (Bg) is stored in seed in large amounts (3% of seed total proteins). It consists of two subunits, one of 27 KDa, the other of 16 KDa encoded by the same mRNA, linked by disulfide bridges. Bg is synthesized as sole precursor polypeptide consisting of a putative peptide signal and of two subunits. This polypeptide is processed to yield the mature dimeric protein. In the genome about four copies of the Bg gene are present (Watanabe and Hirano, 1994).

This protein is mainly located in the seed embryonal tissues and its expression pattern is unusual for a storage protein. In fact, a portion of Bg is accumulated in the intercellular spaces of the cotyledon parenchyma (Nishizawa et al., 1994), whereas at an intracellular level it is stored in protein bodies on the middle lamella of cell wall and in the plasma membrane (Watanabe and Hirano, 1994). This location suggests that the Bg is not a mere storage protein, having other functions as well. More accurate data on Bg location and expression period in Soya are not available. It has never been verified whether the site- and time-specific expression mechanism be preserved in other transformed vegetal species (like tobacco). To this end, reference is made to

general data on storage proteins and to studies on Bg homologues in lupine (conglutyn γ), with which it has a high sequence homology. This protein is stored only in lupine embryonal tissues (cotyledons and embryonal axis) 40 days after blooming. It has not been detected in other tissues such as leaves and sprouts. In seeds of transgenic tobacco, the conglutyn γ gene is increasingly expressed from the 15th to the 20th day after blooming until the 40th, then begins to decrease (Ilgoutz et al., 1997).

One of the peculiar features of the Bg is that it is secreted in large amount from Soya seeds soaked in water at 40-60°C. It is uncertain whether the secreted proteins are neosynthetized after heat-treatment, or instead are the proteins already present to be secreted. Since a post-heating increase in specific mRNA has been highlighted, it is assumed that the Bg is actually synthesized as a consequence of the thermal shock (Hirano et al., 1989).

Not much is known on the regulation mechanism of the expression of the gene encoding the Bg protein, nevertheless sequences in the promoter region involved in the gene regulation have been identified. Besides the CAAT and TATA box sequences, respectively located at -116 and -25 with respect to the transcription start site, three regulatory elements similar to thermo-specific sequence enhancers present at the non transcribed 5' region of genes in other organisms, have been detected. These heat shock elements (HSE) consist of two 5 pb conserved units: 5'-NGAAN-3' and 5'-NTTCN-3'. In the thermoregulated promoter of the Soya heat-shock protein, the enhancer elements, observed also in Bg as well, cooperate synergistically with three CCAAT box sequences located upstream thus increasing gene expression; these putative sequences are present also in the bg promoter.

Sequences responsible of the site- and time-specificity expression were not identified.

The interest for this protein derives from the fact that it is accumulated in high amounts in the Soya seed (3% of total proteins) and therefore has a strong seed-specific promoter which can ensure a high level of expression of the gene it controls. Moreover, it is known that the regulation mechanism of this protein is different from that of the other storage proteins of Soya seed but the details are not known. However, studies on the promoter and on its site- and time-specific activation mechanism have never been carried out using reporter genes in transgenic plants.

Both Bg and CONG, as storage proteins, are synthesized exclusively in the seed tissue and are stored in large amounts in cells constituting this organ, inside specific compartments. Concerning the post-transcriptional and above all the post translational regulation level, it runs through the mechanisms of intracellular transport and of protein compartimentalization, which are to date to be clarified in many aspects.

In fact, those mechanisms involve all processes influencing the concentration, retention and distribution of the proteins in the endomembrane system (Okita, 1996).

However, general principles of protein targeting do exist, valid for all plant species.

1. Targeting information are contained in the proteins themselves, as discrete signals. Those signals are intercepted by specific recognition signals such as receptors or simple interactions with membrane lipids.

2. Different types of signals do exist (topogenic sequences) each with a specific function. Among them there are signal sequences that start the protein translocation across specific endomembranes and interact with receptors/translocators facilitating the unidirectional transfer. Then there are stop and retention sequences that block the transfer to the membrane or to the inside of the compartment. Selection

sequences target proteins to the various cellular compartments. All those elements can be of a sequential type, i.e. localized in the N-terminus, central or C-terminus portion of the protein, or conformational, i.e. consisting of amino acids which although nonsequential, are yet adjacent in the native protein conformation. Moreover, there may be various signals simultaneously, and they can be modified or activated (e.g. by phosphorylation). After transfer the signal is often deleted using specific cleavage sites for endogenous proteases.

mRNA accumulation in a particular region also influences the protein location. Soya seed storage proteins, globulins as well as lectins, are stored in storage vacuoles. In fact, several types of vacuoles do exist. Some of them, besides having the function of maintaining the turgor pressure and of regulating ion, sugars and amino acid release, also constitute the depository of storage and defense proteins. The specific signal sequence for targeting to the vacuole has not been identified yet, apart from some plant species (Kermode, 1996). Probably, one or more surface regions of the correctly conformed protein are recognized by the selection mechanism. Plant cells possess the unique feature of accumulating storage proteins in the protein bodies, whereas in animals similar inclusion bodies are formed only when an excess of protein synthesis occurs. Therefore, the latter protein bodies consist of unmuddled accumulation of conformationally incorrect or partially processed proteins. The formation and organization process of the protein bodies in plants remains unclear, although it is known that it consists of a series of ordered events (Okita, 1996). Globulins are proteins with an acidic isoelectric point (pI), accordingly they are translocated in the endoplasmic reticulum (ER) and in the Golgi complex as soluble proteins. As soon as they reach the vacuoles, due to their low pH or possibly to the

processing and assembly, these proteins precipitate forming particled aggregates that will thereafter originate the protein bodies (Kermode, 1996).

In leguminosae, different storage proteins are accumulated in the same protein bodies with no spatial segregation. In other plant species the protein bodies form in the ER and are then absorbed in vacuoles by autophagocytosis (Kermode, 1996).

This general pattern is well-grounded for β-conglycinine as well, though the specific vacuole targeting sequence have not been identified for this protein. Instead, the β-conglycinine binding with a BIP-homologous protein has been observed. This protein functions as chaperonine and, just like other proteins in different plant species, can have the role of retaining β-conglycinine in the ER until its correct conformation is reached (Galili et al., 1993; Shewry et al., 1995; Kermode, 1996; Fontes et al., 1996). As for 7S basic globulin, available information is scarce. It is known that it is located in protein bodies on the middle lamella of cell wall and in plasma membrane and not in vacuoles (Watanabe, 1994). For this reason, its mechanism of division into compartments is hypothesized to be different from that of β-conglycinine. However, it is known that even wall-located proteins follow the same transport pathway of the vacuole proteins, i.e. are translated in the ER, then transported to Golgi and lastly secreted to the outside or inserted in the membrane by vesicular traffic.

However, storage proteins expression in heterologous hosts shows that the compartimentalization mechanism is universal. In transgenic plants the seed vacuole storage proteins are correctly targeted. Nevertheless, sometimes transport can be inefficient, especially in vegetative organs with respect to seeds. In tobacco, leguminose storage proteins are correctly targeted to vacuoles both in seeds and in leaves, yet in leaves there is a lower

accumulation level. This is so because a difference exists in the transportation efficiency or because of a different processing rate (different proteases or higher instability). Hence, it can be understood how seed-specific in plant production of a heterologous human protein is a complex mechanism, so that the preliminary verifying of the functioning and efficiency of the expression system, as constructed in a model host organism, constitutes an important step.

It has been seen how tobacco is one of the most widely used plants to this end. Its preferential use in assays derives from the fact that it is one of the better known plants, both in a genetic and in a biological and physiological respect. This, together with the ease of effecting the genetic transformation and the shortness of vegetative cycle, made it become an important model for biotechnological experiments, a model whose transformation specific systems and micropropagation conditions are now better known. Additionally, tobacco possesses the further advantage of a near-complete extensibility of the obtained results to several other plant species, consequence of the high conservation rate of genic control mechanisms, that precisely proved to be usually highly conserved in other plant species, and in particular in leguminosae. Therefore it is particularly suitable for the study of the promoters taken out therefrom, and in particular of their capacity of allowing a gene of interest to be expressed in a controlled way in a transformed plant. Genes of interest are usually those encoding proteins suitable in a pharmaceutical or alimentary field. Accordingly, a heterologous gene of interest for this kind of application is that of the human lactoferrin, a protein belonging to the transferrin family, and as such capable to stably and reversibly bind two iron ions.

In fact, by virtue of its biological functions lactoferrin turns out to be interesting from a

nutritional as well as from a pharmacological point of view. It is present in human milk and has a fundamental role in neonatal feeding, as a matter of fact several biological functions have been attributed to this protein, among which a bactericidal and bacteriostatic activity against a wide range of pathogenic microorganisms and the capacity of increasing iron absorption at the intestinal level (Lonnerdal and Iyer, 1995; Hambraeus and Lonnerdal, 1993). Moreover it promotes cellular growth, controls myelopoiesis and is capable of modulating the inflammatory response (Lonnerdal and Iyer, 1995; Oguchi et al. 1995; Penco et al. 1995).

Therefore, at first, attempts to research in milk of other mammals a protein capable of binding iron and possessing the same properties were carried out.

It has been observed that milk of all mammals contains two types of iron-binding proteins, present in different ratios: transferrin, identical to serum transferrin, and lactoferrin. Human milk has a particularly high lactoferrin content, in fact its concentration in colostrum is of 5-10 mg/ml, although it decreases during lactation to about 1 mg/ml in ripe milk (Hambraeus and Lonnerdal, 1993). However, the amount of lactoferrin is much lower in milk of other animal species, like goat, horse, pig and mouse, In cow's milk for instance its concentration is of about 0.1 mg/ml. In some species such as rabbit, rat and dog, lactoferrin is absent, the prevailing iron-binding protein being instead transferrin.

Further, lactoferrin (LF) produced by other non-human mammal species, assumes in each of them different structural characteristics, and therefore different properties.

Human lactoferrin (LFU) is a 78 KDa monomer glycoprotein, with a bilobate structure. There is a high degree of homology between the N-terminus domain and the

C-terminus one, both at the amino acidic sequence (37%) and at the tridimensional structure level. The tridimensional structure has been described in detail by X-ray crystallography (Lonnerdal and Iyer, 1995). The gene encoding LFU has been cloned and sequenced. Genic control mechanisms at a transcriptional and translational level and estrogens and iron role in those mechanisms are also known (Liu et al, 1991). The mature protein consists of a 692 aa polypeptidic chain with a 8.8 - 9 pI. It contains 16 disulfide bridges and shows some resistance to proteolysis (Lommerdal, 1995), has three glycanic polyacetylactosaminic chains bound with N-glycosidic bonds to the amino acidic residues Asn233, Asn476 and Asn545 and the molecular weight of the glycosilated protein is 82 KDa.

One of the most important differences existing among lactoferrins (LFs) present in the various animal species is precisely the glycosidic chain composition. In fact, unlike human LF, bovine LF contains α-1,3 galattosidic residues and glycans of oligomannosidic type; the role of the glycosidic chains has not been defined yet, however it is possible that glycans protect LFU against attacks from proteolytic enzymes. Each of the two LFU domains is capable of binding tightly, yet reversibly to a ferric ion and at the same time to a carbonate or bicarbonate ion molecule (Hambraeus and Lonnerdal, 1993). Iron binding sites in human milk lactoferrin are not completely saturated, but only at 6-8% of their capacity (Stowell et al., 1991).

In recent years, a series of studies aimed at understanding the mechanisms of action and the relation between molecular structure and function of this protein have been carried out. The strategy adopted was that of studying LF molecules structurally altered by site-specific mutagenesis. Accordingly, expression systems of LFU recombinants producing in a simple and inexpensive way a protein as identical as possible to the one

purified from human milk were carried out.

However, all heterologous hosts used so far for the LFU recombinant are eukaryotes, as, it being a complex glycoprotein, requires a sophisticated processing apparatus.

In 1991 Stowell et al. cloned the LFU gene in cultured neonate hamster renal cells. An inducible $Zn^{2+}$-promoter and the secretion signal of a hamster endogenous protein were used to maximize expression. Concentration of LFU recombinant secreted in the culture medium was of about 20 mg/l, sufficient for crystallization and therefore for structural studies. Characterization revealed that it has the same molecular mass of native LFU maintaining intact the iron-binding site. It only differs from human milk LFU in the glycosidic chains and N-terminus sequence, but these do not influence folding. Such an expression system is highly expensive and not suitable for the production of the amounts of proteins required at an industrial level.

Then LFU was expressed in transgenic mice's milk. In this case the entire animal rather than the cultured cells was transformed, using the regulation sequence of the bovine gene α-S1 caseine. It was shown that LFU mRNA is exclusively expressed in female mammary gland during lactation. In milk the protein reaches a 0.1-36 µg/l concentration. Recently this LFU recombinant has been characterized (Nuijens, 1997), and it has been observed that it has the same molecular mass, N-terminus sequence and immunoreactivity of native LFU. It also maintains the capacity of releasing iron at acidic pH and the bond to bacterial lipopolysaccharides. Also in this case, glycosilation, as well as in vivo bactericidal and antiinflammatory action, are different from the ones in native LFU.

Indeed, a highly significant production system of recombinant LFU is that carried out in Aspergillus awamori (Ward et al, 1995). This method, which is

patented, yields commercial amounts of recombinant LFU (2 g/l). In order to maximize expression LFU is produced as a fusion protein with part of the structural gene, regulation sequence and secretion signal in the culture medium of the glucomylase. The fusion polypeptide is processed to yield mature LFU by an endogenous peptidase. Glucoamylase is an Aspergillus protein expressed in high amounts.

An alternative eukaryotic host is the one used by Mitra and coworkers in 1994. They have transformed tobacco cells in suspension. In the transgenic calluses a protein much shorter than the native LFU and therefore found to be unstable is produced in small amounts. Recombinant LFU shows activity against phytopathogenic bacteria, e.g., Xantomonas campestri, Pseudomonas syringae and others. In the above-mentioned study the obtaining of entire and fertile plants is not reported.

Recently LFU was also produced in culture in insect cells, using Baculovirus as expression system (Salmon et al., 1997). This is a highly powerful expression system , yielding a recombinant protein identical to the native one, apart from the glycosilation level. Nevertheless it maintains the binding with the specific receptors.

All the above reported expression systems allow to obtain an amount of protein sufficing for functional studies, and in some cases (Aspergillus) for commercial uses as well. In the latter case however, safe use of the purified protein, e.g. in milk substitutes for neonates, requires an excessive purification in order to ensure the absence of immunogenic or allergenic substances. However, transgenic plants have never been used to this end, nor products directly suitable in human nutrition have been ever yielded, for instance by the recombinant LFU expression in alimentary plants.

Summary of the invention

The present invention relates to a general system of tissue-specific, and in particular seed-specific,

accumulation of heterologous proteins, designed and carried out with the object of maximizing the production while preventing the degradation thereof, by using leader sequences and promoters of Bg and β conglycinine genes. To this end, the structural part of the selected gene may encode proteins having an enzymatic activity, used in human therapy or in industrial processes, or proteins with a general (lactoferrin) or specific (antibodies or antigens) pharmacological activity, or antibody proteins for phytopurification or for the elimination of mycotoxins present in foods.

The present invention also relates in particular to a system that, enabling the in plant tissue-specific expression of the human lactoferrin gene, provides an important solution to the problem of the production of this protein. This system in fact determines the production of plants capable of expressing relatively high amounts of this protein that, in the preferred embodiment providing the expression of a synthetic gene designed by the inventors so as to maximize its in plant expression, reaches industrially relevant levels. Moreover, such transgenic plants allow to avoid the costly product purification processes, as they can be used as *nutraceuticals*, and therefore being directly intaken as alimentary products. Accordingly, also the use for the production of protein flours or of protein extracts yielded from tissues, and specifically from seeds of the afore mentioned transgenic plants, for the production of functional foods or special preparations for children is possible.

The protein accumulation in the seed further allows to significantly increase the iron content of the same, or of the flours derived therefrom. Accordingly this system allows the obtaining of foods which, having a twofold iron content with respect to analogous product, can be also used for supplying this micronutrient's deficiency through a normal alimentation.

These plants can anyhow be used also for human lactoferrin purification, by conventional methods based on chromatography techniques.

This expression system further provides the use of new recombinant vectors, constructed by testing the effect of various leader sequences and processing sites of the mature protein, enabling the production of any protein of interest and in particular of lactoferrin or of fragments derived thereof, with a tissue-specific protein accumulation, in plants belonging to different families among which leguminosae, cereals, solanaceae, fruit-bearing plants and horticultural produce in general. In particular they are structured so as to have the following functionally linked components: (a) a promoter; (b) a signal sequence; (c) a nucleotide sequence optimized for in plant expression and corresponding to the amino acidic sequence of the entire human lactoferrin or to fragments thereof; (d) a polyadenylation signal.

In particular the case concerning plasmid is considered, and wherein regulation elements and signal sequences used are those belonging to two genes encoding storage proteins that are very common in Soya seeds, i.e. a β-conglycinine and a 7S basic globulin, isolated and cloned from the Richland soya variety. They can be used to transform plant cells both by the Agrobacterium method and by direct physical methods (Gelvin and Schilperoort, 1995; DuPont Biolistic Manual, DuPont). The vegetal transformed cells are hence selected with the selection agent provided for the purpose and induced to form entire fertile plants capable of forming seeds, in turn capable of expressing the gene for lactoferrin, and accumulating it as storage protein.

This result was obtained by designing and synthesizing an artificial gene encoding the same amino acidic sequence of the natural human gene, but having sequence mutations such that codons most frequently used

by the human cell are replaced with those most frequently used by the vegetal cell. This result yielded the change of the 31% of the codons in the original gene (see table 1). The remarkable production of human lactoferrin detected in the various plants transformed with the synthetic gene, with yields going from 1% to 1.8% of the seed total storage proteins, but not in plants having the natural gene, proved the in plant functionality thereof. Furthermore on the basis of such sequence, which allows the efficient expression of the human lactoferrin in any vegetal cell, a person skilled in the art can derive the specific sequence expressed with a higher efficiency in each single vegetal species, by the simple application of the common general knowledge.

Concerning all the above disclosed, object of the present invention is a polynucleotide encoding human lactoferrin, characterized in that it has a sequence totally or partially corresponding to the sequence reported as SEQ ID NO:1, or to a sequence biologically equivalent thereof and in that said sequence is optimized for in plant expression, and in particular the case wherein said polynucleotide has fused to the 5'-terminus end a sequence selected from the group comprising the sequences shown as SEQ ID NO: 13 and NO: 14. A particular case of sequence partially corresponding to the SEQ ID NO:1 is given by a sequence corresponding to one or more fragments of said sequence.

Object of the present invention is also the human lactoferrin protein, obtained from the expression of the afore mentioned sequences.

A further object of the present invention is a recombinant DNA vector, in particular a plasmid, comprising at least one sequence of a gene of interest, in particular the gene encoding the complete human lactoferrin, specifically a sequence totally or partially corresponding to the SEQ ID NO:1, operatively linked to regulation elements enabling the tissue-specific

expression of said gene. A special case is the one where such regulation elements consist of an expression cassette for plants allowing tissue-specific expression of said gene.

Further cases of interest are those wherein the expression cassette for plants are constituted by the regulation elements of the protein 7S basic globulin, and in particular when among said regulation elements there is the sequence reported as SEQ ID NO:21, or the regulation elements of the β-conglycinine protein, and in particular when among said regulation elements there is the sequence reported as SEQ ID NO:22, the case wherein the sequence of the gene encoding complete or partial human lactoferrin is operatively linked, or even fused, to a leader sequence, and the case wherein such leader sequence is selected from the group comprising sequences SEQ ID NO: 13 and NO: 14.

Of particular importance is the case wherein such plasmid is selected in the group comprising vectors pBI, pGEM or pUC.

A further object of the present invention is constituted by the transformation process of vegetal cells wherein the transformation is effected with one of the above-mentioned vectors, the transgenic vegetal cells can be obtained through transformation of wild type vegetal cells with at least one of the same vectors, and by the cells that anyhow contain a gene of interest, or portions thereof, and in particular the gene encoding human lactoferrin operatively linked in an expression cassette, enabling the tissue specific expression of the gene itself, in particular that of the gene encoding 7S basic globulin and that of the gene encoding β-conglycinine.

A further object of the present invention is constituted by cellular aggregates and in particular calluses characterized in that they are obtainable by the aforesaid cells.

A further object of the present invention are also the transgenic plants obtainable from the aforesaid cells with conventional techniques, or anyhow containing a gene of interest and in particular the gene encoding human lactoferrin, specifically the one with a sequence corresponding totally or partially to SEQ ID NO:1, operatively linked in an expression cassette enabling the tissue specific expression of the gene itself.

Of particular relevance is the case wherein such transgenic plants are selected from the group comprising solanaceae, cereals, leguminosae, horticultural produce and fruit-bearing plants in general, in particular Soya, tobacco and rice, wherein the gene encoding lactoferrin is specifically expressed in the storage tissues or in the fruit. A further object of the present invention is the use of such transgenic plants as *nutriceuticals*.

A further object of the present invention is constituted by the production processes of functional foods containing proteins produced by the aforesaid transgenic plants, of vegetal milks, starting from natural and/or concentrated proteins deriving from the above-mentioned plants, and anyhow any human lactoferrin production process, characterized in that it utilizes the aforesaid plants.

Lastly, object of the present invention is also the human lactoferrin obtained from the aforesaid transgenic plants.

The invention will be better described with the aid of the annexed figures.

Description of the figures

Figure 1 shows the strategy adopted for the assembly of the synthetic gene encoding human lactoferrin.

Figure 2 shows the map of plasmids pGEM-PGLOB(A) and pGEM-PCONG (B) obtained from the cloning of the Soya promoters in plasmid pGEM (Promega), in which the restriction sites used to derive the plasmids are highlighted.

Figure 3 shows agarose gel electrophoresis analysis of digestion of plasmids pGEM-PGLOB with Sal I (lanes 1, 2, 3 and 4) and pGEM-PCONG CON Sph I (lanes 6, 7, 8 and 9), carried out to test clockwise orientation of the insert. All PGLOB samples tested positive, yielding fragments of the expected sizes. PGLOB 1 is sample 1 selected for the subsequent molecular work. In contrast, PCONG samples did not yield the expected pattern, suggesting the possibility of the presence of errors due to the adopted cloning technique (hypothesis later discarded, see figure 6) or due to isolation of a variant of the control region that in the Richland variety differs from the one of the disclosed sequence (Dare variety). Sample 5 is lambda marker HindIII.

Figure 4 shows agarose gel electrophoretic analysis of the restriction pattern of two plasmid pGEM-PCONG clones with enzymes Nde I (lanes 1 and 2), Rsa I (lanes 3 and 4), and SnaB I (lanes 9 and 10), carried out in order to test orientation and identity of the constructs. Cuts with Nde I, Apa I and SnaB I yielded the expected patterns in contrast to the cut performed with Rsa I; this results are justified from the differences found in sequence and reported in figure 6. In lanes 5 and 6 markers of molecular weight λ-DNA Hind III and Marker IV (Boehringer) respectively are present.

Figure 5 shows electrophoretic analysis of the restriction of various clones of plasmid pGEM-PCONG with Rsa I (lanes 1-6) and Hinf I (lanes 9-14) enzymes in order to test identity of constructs. In both cases the obtained pattern do not mirror the expected ones, but are conserved among the different clones, thereby suggesting their being due to differences in the original sequence with respect to the published restriction map and not to errors in the amplification phase with Taq polymerase or in the cloning. Adopted markers are λ-DNA Hind III and Marker IV.

Figure 6 shows the comparison between the published

sequence of gene CONG promoter region and the one cloned in plasmid pGEM-PCONG.

Figure 7 shows a schematic view of the two plasmids resulting from the cloning of the native gene LFU into the two vectors pGEM-T and pBI121, carried out in order to obtain plasmids used later on as transformation control.

Figure 8 shows the map of the two plasmids resulting from synthetic LFU gene cloning into vectors pGEM-PGLOB and pGEM-PCONG, i.e. plasmids pGEM-PGLOB-LFU (A) and pGEM-PCONG-LFU (B), respectively.

Figure 9 shows the map of plasmids pBI-PGLOB-LFU (A) and pBI-PCONG-LFU (B) wherein the restriction sites used are highlighted. In particular, box (A) shows the construction of a plasmid containing the synthetic gene represented in the sequences list as SEQ ID NO:1 and cloned in plasmid pBI101 fused to promoter PGLOB and in Open Reading Frame with the "leader" of 7S basic globulin.

In contrast, box (B) shows the construction of a plasmid containing the synthetic gene reported in the sequence list as SEQ ID NO: 1 and cloned in plasmid pBI101 fused to promoter PCONG and in open reading frame with the β-conglycinine leader.

Figure 10 shows electrophoretic analysis of the restriction of various clones of plasmid pBI-PCONG-LFU with enzymes Xba I, BamH I, Sac I; samples 4 and 5 test positive. In position 6 molecular weight marker Ladder 1Kb is present.

Figure 11 shows electrophoretic analysis of the restriction of two clones of plasmid pBI-PCONG-LFU with enzymes Sal I (lanes 1 and 2) and with Xba I and Sac I (lanes 4 and 5); Both samples tested positive. Samples 3 and 6 represent the positive controls, i.e. pGEM-PCONG-LFU digested with Sal I, Xba I and Sac I respectively.

Figure 12 shows agarose gel electrophoresis analysis of PCR products from genomic DNA extracted from various

plants transformed with pBI-PGLOB-LFU, performed using primers PLT48 and PLT49 for the promoter sequence PGLOB. Positive samples 2, 3, 4 and 5 represent the band of the DNA amplified to 1500 base pairs, while samples 6, 7 and 8 represent the negative control of PCR and the positive control (pGEM-PGLOB) respectively. Molecular weight markers Ladder 1Kb are found at 1 and 9.

Figure 13 shows in box (A) the agarose gel with the genomic DNA of tobacco transformed with pBI-PCONG-LFU (lanes 1-5) or with pBI-PGLOB-LFU (lanes 6-9) cut by enzyme BamH I. M is the molecular weight marker Ladder 1 Kb. Sample 10 shows the positive control pGEM-LFU, not shown in the photo for quantitative reasons. In box (B) the hybridization pattern of human LF on the genomic DNA of the same tobacco plants is shown; samples 1, 2 and 3, belonging to plants PCONG 1, PCONG 3, and PCONG 4 respectively are positive, as is the case for samples 5, 7 and 8, belonging to plants PGLOB 10, PGLOB 3 and PGLOB 4 respectively. It is evident that pGEM-LFU, the positive control (lane 10), was only partially digested as also the super-coiled plasmid forms are present.

Figure 14 shows SDS-PAGE electrophoretic analysis of proteins partially purified from seeds of the transgenic plants tested with Southern analysis of the preceding figure (A) and Western analysis of the same proteins, after transfer to a membrane, using polyclonal antibodies specific for the human lactoferrin (B). In particular, in box (A) SDS-PAGE electrophoretic analysis of total cellular proteins (30 DAP) from mature seeds of transgenic tobacco is shown. In position 2, 3, 4 and 5, 6, 7 the same samples tested positive to Southern analysis, extracted by buffer at pH 2.7 and pH 7.6 respectively, are found. Samples 8 and 10 represent the positive control (milk-extracted human lactoferrin, Sigma) and the negative control (non-transformed plant of the same variety), while in position 9 the molecular weight marker Rainbow (Amersham) is found. In box (B)

autoradiography of anti-lactoferrin antibody hybridization with the same proteins transferred to DEAE-nitrocellulose membrane is shown; the sample corresponding to plant PGLOB 10, in position 2 and 5, does not yield a positive signal, although according to Southern analysis it is transformed. All other samples are positive.

Figure 15 shows electrophoretic analysis of raw proteins extracted from seeds and leaves of the transgenic tobacco transformed with the plasmids of which at figure 9. In particular, in box (A) protein coloration carried out with Coomassie blue is reported. In box (B) Western Blotting carried out with human LFU-specific antibodies on the proteins of the gel shown in box (A) after transfer to membrane is reported. In particular, in lane 1 plant PGLOB 1, with leaf-extracted proteins is reported, in lane 2 always plant PGLOB 1, with seed-extracted proteins is reported, in lane 3 plant PGLOB 3, leaf proteins, is reported, in lane 4 plant PGLOB 3, seed proteins, is reported, in lane 5 plant PCONG 105, leaf proteins, is reported, in lane 6 plant PCONG 105, seed proteins is reported, in lane 7 plant PCONG 105, seed proteins treated with N-deglycosilase F, is reported (see text), in lane 8 the molecular weight marker is reported, in lane 9 the human LFU present on the market treated with N-deglycosilase F is reported, in lane 10 LFU present on the market is reported.

Figure 16 shows Western analysis of LFU protein extracted from human milk and of recombinant protein isolated from tobacco seed, before and after N-deglycosilase F enzyme treatment. Analysis was performed with human lactoferrin specific antibodies. In lane 1 LFU extracted and purified by HPLC from seeds of plant PCONG 105 is reported; in lane 2 a protein as in 1 after a 18-hours treatment with N-deglycosilase F is reported; in lane 3 commercial LFU after a 18-hours treatment with N-deglycosilase F is reported; in lane 4 commercial LFU is

reported, a diminution is apparent in the molecular weight of the two enzyme-treated samples (2 and 3).

Detailed description of the invention

The strategy adopted for the generation of transgenic plants capable of producing human lactoferrin was developed along two directions: on the one hand, comparative analyses on plant expression systems, particularly tobacco and Soya, have been carried out, in order to have a basis for the designing of a sequence encoding human lactoferrin thereof, sequence optimized to maximize its expression in vegetals. Accordingly in the sequence designing the necessity that the required post-translational modifications for the production in the active form could be effected on the translated protein, and that, both for its conformation and due to its subcellular localization, the protein be sufficiently stable to be accumulated in relevant amounts in the transformed plants, was taken into account. This proved crucial, having ascertained after various attempts carried out in the past years the impossibility of an in plant production of human lactoferrin using constitutive expression systems (e.g. promoter 35S) as well as promoters inducible by leaves cut. Moreover, besides difficulties related to the type of promoter used, the production level and the stability of the protein were tested to be scarce and depending on a warped preferential use of the codons between the human gene and the plants.

Therefore, a plasmid vector system was developed utilizing vectors containing a newly synthesized lactoferrin gene regulated by tissue- and stage-specific promoters capable of yielding a high gene expression and of accumulating the protein in a stable and efficient way inside seed storage organs. Moreover the selection of leader sequences and the design of the fusion point between those and the structural portion of the mature protein yielded a lactoferrin protein that, in

quantitative and possibly also in qualitative terms, has the same glycosilation level and the same amino terminal sequence of the native protein, which is important for some of its functional characteristics.

Concerning the synthetic gene design, all the necessary and possible triplets were modified taking into account their preferential use in the two reference plants, tobacco and Soya. In particular, data represented in table 1 were used.

TABLE 1

| CODONS | | HUMAN | SOYA | TOBACCO | LEU WT | LEU SYN |
|---|---|---|---|---|---|---|
| ARG | CGA | 5.5 | 4.5 | 4.8 | 3 | 3 |
| | CGC | 11.2 | 7.5 | 3.8 | 5 | 5 |
| | CGG | 10.7 | 2.2 | 2.2 | 7 | 3 |
| | CGT | 4.5 | 7.4 | 7.7 | 4 | 8 |
| | AGA | 9.6 | 14.8 | 12.5 | 13 | 16 |
| | AGG | 10.8 | 11.3 | 12.1 | 13 | 10 |
| LEU | CTA | 6.1 | 7.0 | 7.4 | 0 | 2 |
| | CTC | 20.1 | 16.2 | 12.7 | 10 | 9 |
| | CTG | 42.1 | 10.3 | 7.1 | 29 | 8 |
| | CTT | 10.8 | 23.7 | 21.8 | 9 | 19 |
| | TTA | 5.4 | 8.4 | 10.3 | 2 | 2 |
| | TTG | 11.1 | 20.5 | 20.7 | 9 | 18 |
| SER | TCA | 9.7 | 14.7 | 15.8 | 5 | 11 |
| | TCC | 17.8 | 9.5 | 10.0 | 13 | 7 |
| | TCG | 4.1 | 4.3 | 4.3 | 2 | 2 |
| | TCT | 13.3 | 17.3 | 20.6 | 10 | 14 |
| | AGC | 18.7 | 16.8 | 9.0 | 13 | 9 |
| | AGT | 9.9 | 14.0 | 11.9 | 8 | 8 |
| THR | ACA | 14.3 | 14.9 | 16.4 | 8 | 8 |
| | ACC | 22.6 | 14.0 | 11.9 | 9 | 7 |
| | ACG | 6.6 | 3.2 | 4.0 | 2 | 2 |
| | ACT | 12.5 | 17.4 | 20.4 | 12 | 14 |
| PRO | CCA | 15.4 | 30.6 | 26.2 | 6 | 12 |
| | CCC | 20.6 | 10.5 | 9.0 | 10 | 5 |
| | CCG | 6.8 | 4.6 | 3.2 | 6 | 4 |
| | CCT | 16.1 | 22.2 | 21.7 | 13 | 14 |
| ALA | GCA | 14.4 | 20.5 | 22.3 | 12 | 18 |
| | GCC | 29.7 | 16.6 | 16.2 | 25 | 16 |
| | GCG | 7.2 | 4.7 | 4.4 | 8 | 8 |
| | GCT | 18.9 | 23.2 | 35.2 | 18 | 21 |
| GLY | GGA | 17.4 | 22.7 | 31.4 | 11 | 21 |
| | GGC | 25.3 | 11.9 | 14.0 | 24 | 8 |
| | GGG | 17.5 | 11.0 | 10.0 | 12 | 8 |
| | GGT | 11.5 | 22.1 | 30.0 | 6 | 16 |
| VAL | GTA | 6.1 | 9.5 | 11.5 | 2 | 3 |
| | GTC | 16.2 | 9.0 | 13.8 | 7 | 6 |
| | GTG | 30.7 | 25.8 | 14.4 | 28 | 18 |
| | GTT | 10.2 | 24.4 | 29.4 | 5 | 15 |
| LYS | AAA | 21.9 | 23.2 | 23.2 | 22 | 22 |
| | AAG | 35.2 | 35.2 | 30.7 | 23 | 23 |
| ASN | AAC | 22.3 | 29.2 | 26.0 | 17 | 17 |
| | AAT | 16.5 | 20.2 | 27.8 | 14 | 14 |
| GLN | CAA | 10.8 | 27.4 | 22.5 | 8 | 14 |
| | CAG | 33.8 | 20.7 | 34.1 | 21 | 15 |
| HIS | CAC | 14.7 | 8.9 | 8.9 | 4 | 4 |
| | CAT | 9.3 | 12.1 | 11.1 | 5 | 5 |
| GLU | GAA | 25.4 | 34.6 | 25.7 | 15 | 18 |
| | GAG | 41.6 | 35.8 | 25.2 | 21 | 15 |
| ASP | GAC | 28.9 | 18.8 | 17.2 | 22 | 16 |
| | GAT | 21.5 | 29.7 | 33.0 | 16 | 22 |
| TYR | TAC | 18.0 | 17.1 | 16.6 | 12 | 12 |
| | TAT | 12.3 | 16.2 | 20.5 | 8 | 9 |
| CYS | TGC | 13.8 | 9.9 | 8.1 | 17 | 17 |
| | TGT | 9.9 | 5.5 | 10.1 | 15 | 15 |
| PHE | TTC | 22.1 | 23.5 | 18.1 | 17 | 15 |
| | TTT | 15.8 | 19.3 | 25.2 | 10 | 12 |
| ILE | ATA | 6.1 | 12.2 | 10.7 | 4 | 4 |
| | ATC | 24.3 | 15.0 | 13.7 | 7 | 5 |
| | ATT | 15.0 | 23.7 | 29.4 | 5 | 7 |
| MET | ATG | 22.3 | 20.1 | 22.8 | 4 | 4 |
| TRP | TGG | 13.7 | 11.5 | 13.7 | 10 | 10 |

In carrying out such operation, the value G+C and A+T of the two systems (human and vegetal), the non-tandem repeat of some triplets that may cause shifts in reading, etc. were also taken into due account.

Synthetic LFU gene was then obtained using primers reported in the annexed sequence listing from SEQ ID NO:8 to SEQ ID NO:12 and from sequence SEQ ID NO: 15 to SEQ ID NO: 20 and following the assembling strategy reported in figure 1, consisting in repeated PCR cycles, using for each cycle different pairs of synthetic primers allowing the gradual elongation and the forming of the final sequence as designed. Similarly on the basis of such final sequence the sequences contained the codons preferably expressed in various species of interest, among which rice, have been obtained.

In parallel, also native LFU gene (wild type) encoding human lactoferrin was cloned, always by PCR technique, starting from *a* cDNA library of human mammalian tissue (Clontech). The gene was recovered in its structural part lacking the signal peptide and the poly-A site and cloned in pGEM-T to form plasmid pGEM-LFU whose map is represented in figure 7. Primers designed for amplification are reported in the annexed sequence listing at sequences SEQ ID NO:2 and SEQ ID NO:3; those added the restriction site BamHI at 5' and the restriction site SacI at 3'. After checking the sequence, which tested identical to the published one, the yielded natural gene was cloned in vector pBI121, on sites BamHI and SacI under control of promoter 35S (see figure 7), this plasmid (pBI-LFU) was then denominated pBI-35S-LFU and used as control in the genetic transformation experiments and in the subsequent molecular and biochemical analysis.

Concerning the preparation of the recombinant vectors containing the elements that allow the tissue-specific expression of the LFU gene in expression cassettes for plants, we proceeded as follows. In order

to obtain the seed-specific expression of the protein the promoters and signal sequences of two genes encoding storage proteins that are very abundant in Soya seeds, i.e. a *β-conglycinine* (CONG) and a *7S basic globulin* (GLOB) were used.

These regulation sequences were isolated and cloned from Soya, Richland variety.

In particular, to clone the two GLOB and CONG sequences PCR technique (PCR = Polimerase Chain Reaction; Innis et al. 1990) was used. In this case genomic DNA extracted from Soya leaves of Richland cultivar was used. Oligonucleotides used for specific amplification are reported in the annexed sequence listing from SEQ ID NO: 4 to SEQ ID NO: 7.

For the GLOB promoter the cloned region includes the entire regulation sequence and the sequence encoding the signal peptide (leader) plus the first codon of the structural sequence, such sequence is indicated with SEQ ID NO: 13 in the annexed sequence listing. For the CONG promoter the cloned region includes the entire regulation sequence and the sequence encoding the signal peptide plus the first codon of the structural sequence, such sequence is indicated with SEQ ID NO: 14 in the annexed sequence listing. For both regulation sequences the most suitable restriction site for insertion proved to be XbaI (TCTAGA), while downstream proved to be BamHI (GGATCC), both absent from the native and synthetic lactoferrin sequence, as highlighted in the following tables 2 and 3.

TABLE 2

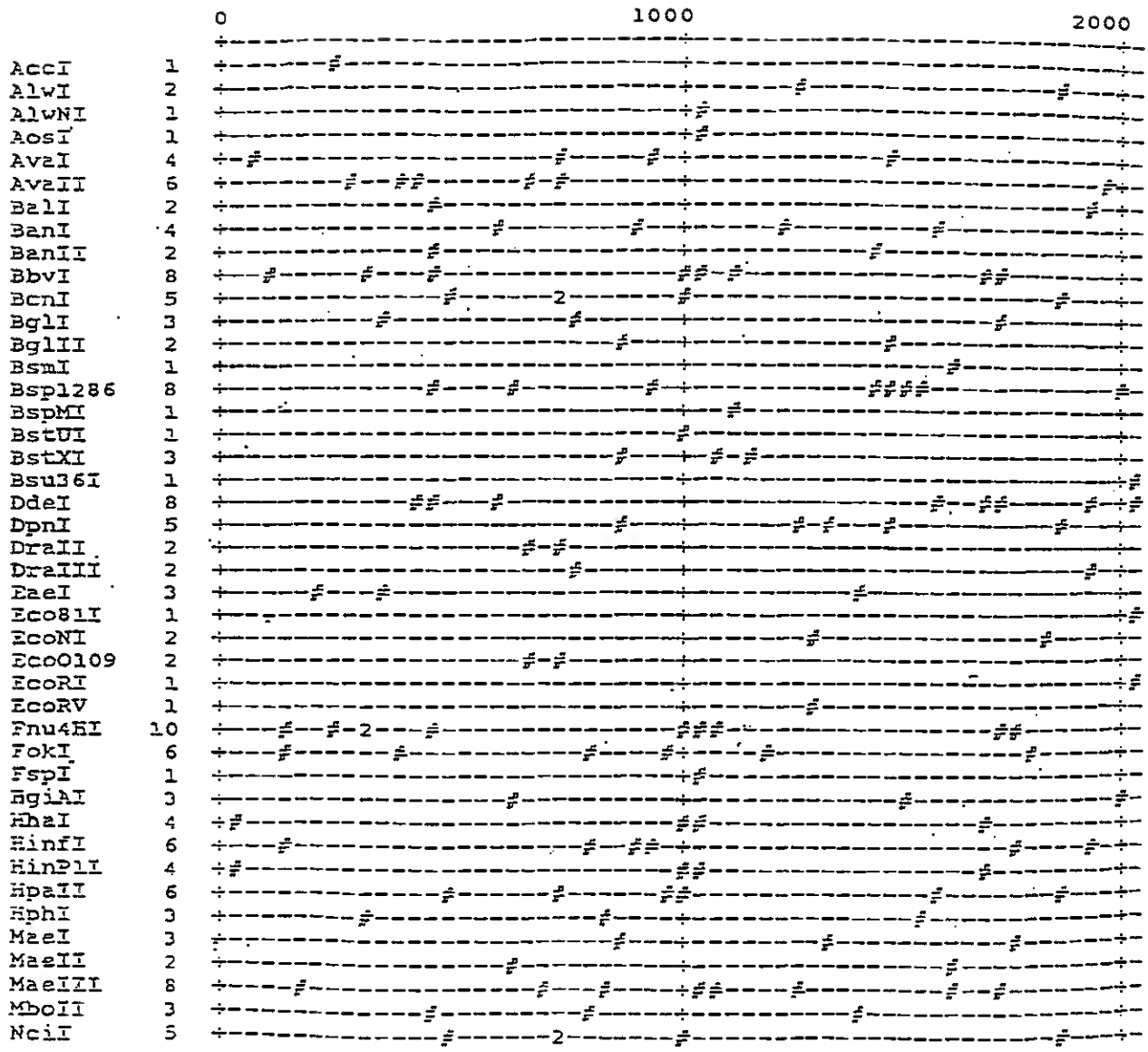

0
1000
2000
AccI 1
AlwI 2
AlwNI 1
AosI 1
AvaI 4
AvaII 6
BalI 2
BanI 4
BanII 2
BbvI 8
BcnI 5
BglI 3
BglII 2
BsmI 1
Bsp1286 8
BspMI 1
BstUI 1
BstXI 3
Bsu36I 1
DdeI 8
DpnI 5
DraII 2
DraIII 2
EaeI 3
Eco81I 1
EcoNI 2
EcoO109 2
EcoRI 1
EcoRV 1
Fnu4HI 10
FokI 6
FspI 1
HgiAI 3
HhaI 4
HinfI 6
HinP1I 4
HpaII 6
HphI 3
MaeI 3
MaeII 2
MaeIII 8
MboII 3
NciI 5

TABLE 2 (continues)

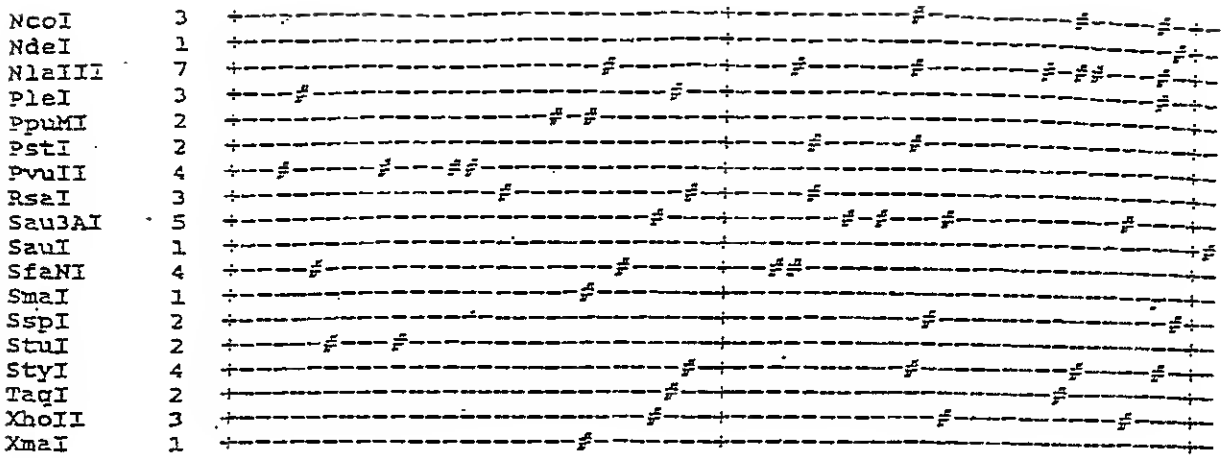


TABLE 3

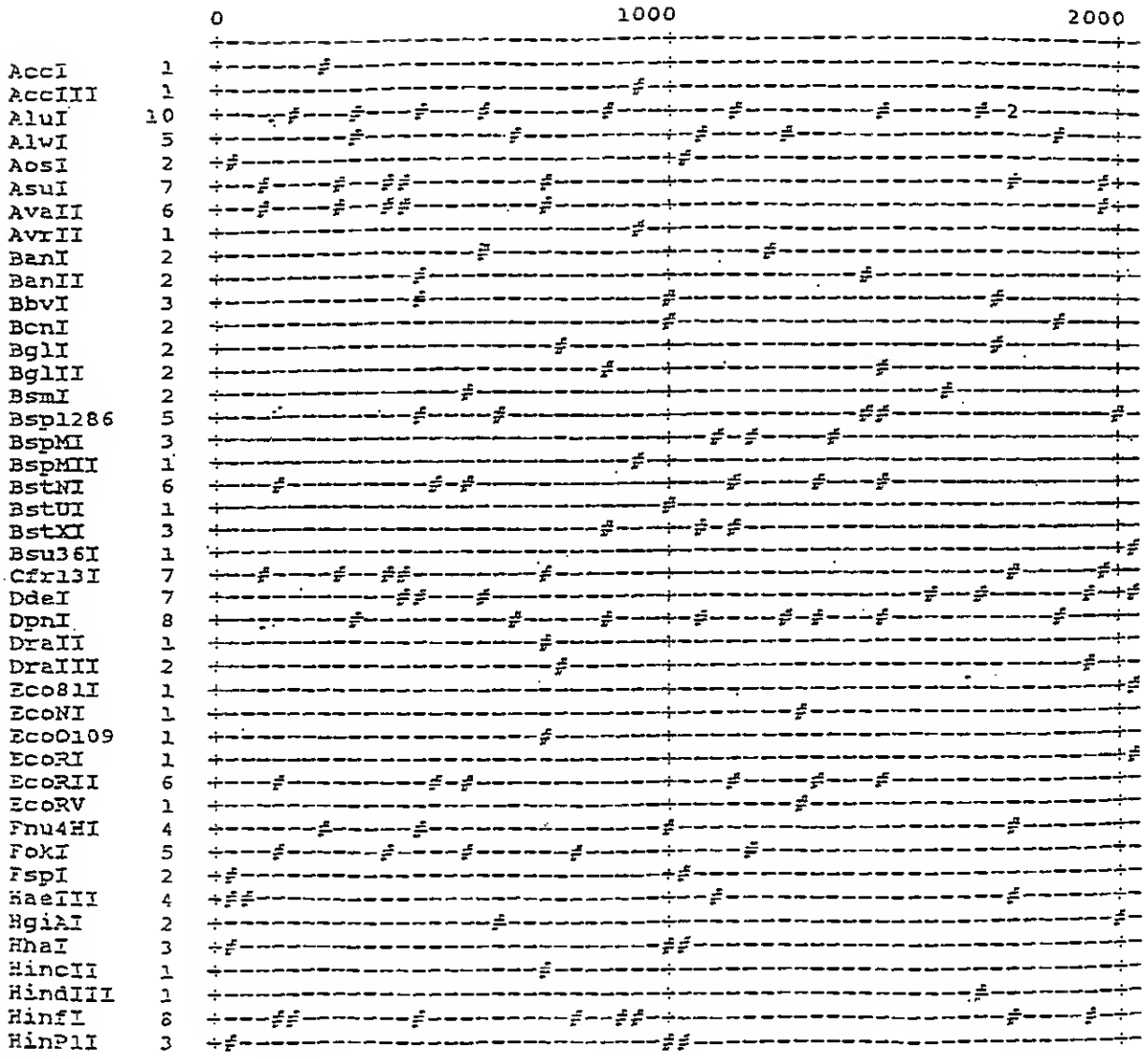
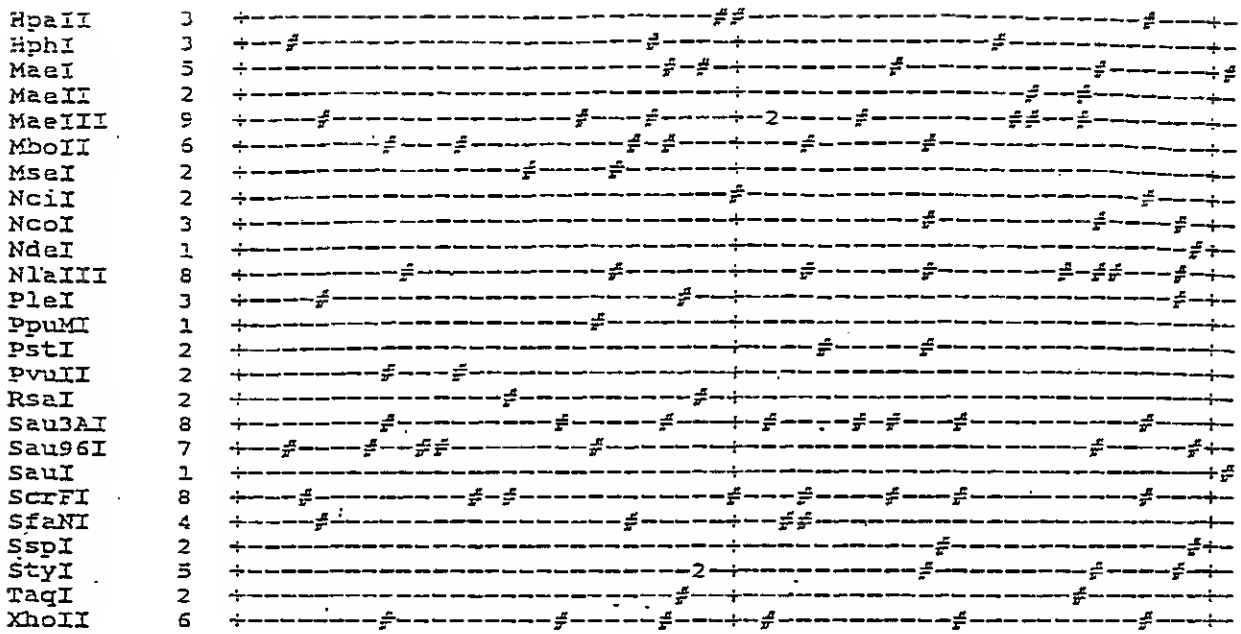

0
1000
2000
AccI 1
AccIII 1
AluI 10
AlwI 5
AosI 2
AsuI 7
AvaII 6
AvrII 1
BanI 2
BanII 2
BbvI 3
BcnI 2
BglI 2
BglII 2
BsmI 2
Bsp1286 5
BspMI 3
BspMII 1
BstNI 6
BstUI 1
BstXI 3
Bsu36I 1
Cfr13I 7
DdeI 7
DpnI 8
DraII 1
DraIII 2
Eco81I 1
EcoNI 1
Eco0109 1
EcoRI 1
EcoRII 6
EcoRV 1
Fnu4HI 4
FokI 5
FspI 2
HaeIII 4
HgiAI 2
HhaI 3
HincII 1
HindIII 1
HinfI 8
HinP1I 3

TABLE 3 (continues)



DNA template was extracted from Glycine max leaves, Richland variety, and amplification products match sizes expected for GLOB (1515 pb) and CONG (1163) promoter on the basis of EMBL sequence data.

Therefore, starting from fragments amplified by ligation in vector pGEM-T, the two vectors pGEM-PGLOB and pGEM-PCONG, whose map is reported in figure 2, were constructed. Yielded plasmids were tested by restriction analysis performed with several enzymes chosen among those cleaving in a limited number and with an overall sequence distribution (see figures 3, 4 and 5) and a clone for each type was selected and sequenced. Sequenced clones showed to be significantly different from the expected sequence. As an example, a comparison between the data bank promoter CONG sequence and the one obtained sequencing clone pGEM-pCONG is reported in figure 6. A 5% difference was detected, therefore the two promoters can be considered as different.

The synthetic gene for human lactoferrin was cloned at first in plasmids pGEM-PGLOB and pGEM-PCONG, cut with enzymes BamHI-SacI, to form plasmids pGEM-PGLOB-LFU and pGEM-PCONG-LFU respectively, whose map is disclosed in figure 8, and then the construct XbaI-SacI transferred in vector pBI101 cut with the same enzymes. In the event of a plant transformation carried out with physical means, as for rice in our case, plasmids pGEM-PGLOB-LFU and pGEM-PCONG-LFU, can be directly used after addition of a terminator, in cotransformation with a vector containing the selection marker (e.g., a PUC-type vector containing the gene for hygromycin resistance). Resulting plasmids pBI-PGLOB-LFU and pBI-PCONG-LFU, whose map is reported in figure 9, were used in the genetic transformation of the plants after an accurate control carried out by restriction with various enzymes to assay the correct integration of the DNA construct (figures 10 and 11).Plasmids pBI-35S-LFU, pBI-PGLOB-LFU and pBI-PCONG-LFU were transferred in A. tumefaciens EHA105 strain cells,

made competent by electrophoresis. Strains containing the three plasmids were used to transform about 450 leaf disks (LD) of tobacco, Petit Avana variety. Formation first of shoots and then of roots was induced from calluses formed on leaf disks (LD) in presence of kanamycin. Once rooted, plants were potted and at least 50 kanamycin-resistant plants were analyzed for each construct. The same plasmids containing, this time, the hygromicyn-resistance genes marker, are used also for the transformation of rice. In this case in particular 40 plants hygromicyn resistant were analyzed.

Plants $T_0$, of rice as well as of tobacco, were tested by PCR technique (figure 12), assaying the presence of the lactoferrin gene inside the genome of the tested plants; plants $T_1$ were assayed by Southern analysis (figure 13), that compared to PCR technique allows a more accurate testing of the transgene presence in the genome, and with Western analysis (figure 14) allowing detection of genic product and therefore the functionality of inserted gene.

All plants with native LFU gene under control of promoter 35S led to accumulation of a protein, recognized by LFU-specific antibodies, of a molecular weight lower than 50 KDa. This protein was found in small amounts in young leaves, becoming undetectable in the fully developed leaves. Plants of rice as well as of tobacco transformed with the two constructs pBI-PGLOB-LFU and pBI-PCONG-LFU produce and accumulate exclusively in seed a protein having a molecular weight of 82 KDa corresponding to the glycosilated human protein as shown by electrophoretic analysis of extracted proteins and by the related Western Blotting carried out with LFU-specific antibodies (see figure 15). Presence of recombinant protein exclusively in the seed and not in the leaves was assayed in all the examined transgenic plants (about 50 for the two constructs) with Western techniques.

Recombinant LFU protein isolated from seed and purified with HPLC technique showed to be identical to the native protein concerning its iron binding capacity and its inhibiting effect towards the examined bacterial strains. Treatment with a deglycosilating enzyme confirms the presence of posttranslational modifications in all alike, at present at least in quantitative terms, to those present in native lactoferrin as highlighted by Western analysis, the results of which are disclosed in figure 16.

Moreover, the contribution of iron consequence of the introduction of the lactoferrin gene in rice plants was assessed. In particular, in the following table 4 results in terms of iron content of some transgenic lines of the Ariete and Rosa Marchetti varieties transformed with plasmids pBI-PCONG-LFU and pBI-PGLOB-LFU are reported. Iron content was measured by atomic absorption after flour mineralization with $HNO_3$ e $H_2O_2$.

Table 4: Analysis of iron content in Ariete (A) and Rosa Marchetti (RM) varieties and of the respectuive transgenic lines capable of in-seed accumulation of protein lactoferrin.

| Sample | Fe Concentration (μg/g) | RSD |
|---|---|---|
| Ariete | 34.3 | 3.0 |
| A cl. 2-3 | 48.5 | 0.7 |
| A cl. 5-1 | 86.5 | 1.8 |
| A cl. 6-2 | 117.0 | 1.2 |
| A cl. 6-3 | 72.4 | 3.2 |
| Rosa Marchetti | 23.5 | 3.6 |
| RM cl. 5-3 | 64.5 | 2.8 |
| RM cl. 2-5 | 76.5 | 2.4 |
| RM cl. 3-6 | 52.7 | 3.0 |
| RM cl. 4-1 | 48.2 | 1.7 |

It is therefore evident from all of the above-reported results that using the native gene, described in the literature, for human lactoferrin under control of the traditional promoters used for genetic transformation

of plants, human protein lactoferrin cannot be produced in relevant amounts, in a stable form and with the posttranslational modifications typical of this protein.

So far a general description has been given of the present invention. With the aid of the following examples, a more detailed description will now be given of specific embodiments thereof, with the purpose of giving a clearer understanding of objects, features, advantages and methods of application of the invention.

EXAMPLE 1:

Agrobacterium Tumefaciens-mediated tobacco transformation

Day 1

A small amount of Agrobacterium tumefaciens of strain EHA 105, taken from a petri plate culture with a sterile loop so as not to exceed in the amount thereby avoiding subsequent problems in controlling bacterial proliferation on plated leaf disks, was inoculated in 2 ml of sterile LB. Then, from a healthy tobacco plant of Petit Avana variety a leaf showing no alteration whatsoever, conversely showing optimal turgor condtions, was taken. The leaf was briefly washed in bidistilled water to remove surface impurities, immersed for 8 min in a 20% sodium hypochlorite and 0.1 % SDS solution and left to dry under a vertical flow hood. From then on all steps were carried out under hood. In particular, the leaf was immersed in 95 % ethanol and shaken in order to completely wet the pages thereof (letting the petiole emerge) for 30 - 40 sec. The leaf was then allowed to dry out completely.

Disks were obtained from the entire leaf surface with an ethanol-sterilized punch, let fall on plates with MS10 free of antibiotics; in particular, the ratio of 30 disks per plate was not exceeded.

Next, 2 ml LB + (just inoculated) Agrobacterium were poured on plate, and the bacterial suspension was evenly spread over the entire plate with a gentle rotatory movement, in order to obtain an homogeneous bacterial

distribution among the disks. LB in excess was carefully aspirated with a pipette. In the course of those steps at all times a parallel negative control was provided by means of a plate to which nothing, or only LB was added.

Then plates were incubated at 28°C for 24-48 hours in constant lighting conditions, and bacterial growth was indicated by the appearance of a thin opaque layer spreading over the entire plate.

Day 2

Leaf disks (=LD) were carefully transferred on a plate with MS10 + 500 mg/l cefotaxime, and incubated at 28°C for 6 days in constant lighting conditions. This step determines the Agrobacterium inactivation.

Day 8

LD were then carefully transferred on a plate with MS10 + 500 mg/l cefotaxime and 200 mg/l Kanamycin, and incubated at 28°C for 14 days in constant lighting conditions. This step determined a selection of the transformed plants: in fact, gene of kanamycin resistance was carried by the plasmid inserted in Agrobacterium.

Day 22

LD, that in the meantime had grown developing a callus, were carefully transferred on a plate with MS10 + 500 mg/l cefotaxime, 200 mg/l Kanamycin and 500 mg/l carbenicillin, and incubated for 6 days. This step determines elimination of the agrobacteria possibly survived to the previous antibiotic treatments (a very frequent occurrence).

Day 28

LD were transferred again on MS10 + 500 mg/l cefotaxime and 200 mg/l Kanamycin, and incubated until shooting. When shoots showed at least two leaves, they were separated from the callous mass and transferred on the radication medium: MSO + 500 mg/l cefotaxime and 200 mg/l Kanamycin.

At the appearance of roots, seedlings were extracted from the plate, freed from agar residues, gently washed

in running water and planted out in loam and sand (2:1) inside small plastic pots. Soil was previously saturated with water, then pots were covered with transparent plastic lids to preserve high humidity conditions, and placed in a growth chamber at room temperature, with a daily 16-hour lighting period.

EXAMPLE 2:

Rice transformation by physical methods

Rice seeds of Ariete and Rosa Marchetti varieties were harvested at milky ripening, when the endosperm is still liquid. The embryo was isolated with a lancet after removal of the two glumes. Immature embryos are of different size and shape, depending on the number of days elapsed from blooming: The ones deemed most suitable for the in vitro culture and the successive transformation, i.e. those of an 1.5 mm average size, were cultured on a medium containing 2,4-D auxine to promote scutellum cell division and suppress differentiation of young embryos, obtaining cellular proliferation of the scutellum area. At this stage embryos underwent bioholistic transformation, with the following parameters: particle size 1.5-3 µm, particle concentration 500 µg, membrane rupturing pressure 1.100 psi, membrane-microcarrier gap 6 mm, microcarrier stop-point gap 6 mm, stop-point target gap 10.5 cm.

Transformation was effected by a cotransformation technique, using as a selectable marker the gene for hygromycin on plasmid pROB5. Cotransformation was effected with a total DNA concentration of 1 µg/µl using 0.6 µg DNA for bombing and with a selectable plasmid/suitable plasmid (pROB5 with pBI-PGLOB-LTU or with pBI-CONG-LTU) ratio of 1:1 assessed on the number of molecules (abt. 1:4 in amount).

Osmotic conditions of the plant material were optimized carrying out a preculture on 3% saccharose and plasmolysis prior to bombing on MS with 10% saccharose for 1 hour. 24 hours after bombing with PDS-1000/He

bioholistic system the material was transferred on 3% saccharose medium.

For selecting the transformational events, bombed tissue underwent selection in presence of hygromycin B (Duchefa). 1 day after bombing embryos were transferred on solid MS medium, additioned with 2 mg/l 2,4-D auxine, 50 mg/l cefotaxime, 50 mg/l hygromycin B, 3% saccharose and 0.35% agarose. One week later embryos were transferred on R2 liquid medium (Ohira et al., 1973) containing 1 mg/l thiamine, 50 mg/l cefotaxime, 50 mg/l hygromycin B and 3% saccharose, pH 5.8, in 190 ml plastic vessels (Greiner). Liquid cultures were shaken at 90 rpm and 28°C in the dark, replacing the liquid medium every 7 days. After 3-4 weeks resistant calluses formed on the embryo surfaces are transferred on R2 solid medium additioned with MS vitamins, 2 mg/l 2,4-D auxine, 50 mg/l cefotaxime, 50 mg/l hygromycin B, 60 g/l saccharose and 0.5% agarose and maintained for 2-4 weeks, until formation of embryogenic structures.

At the formation of embryogenic structures calluses were transferred on solid MS regeneration medium containing 2 mg/l BAP, 0.2 mg/l NAA, 3% maltose, 50 mg/l hygromycin B, 50 mg/l cefotaxime and 0.8% agarose.

Embryogenic calluses were maintained in phytotron at 28°C with 16 hours of lighting to induce formation of shoots, that, once formed, were transferred on hormone-free 1/2 MS radication medium, 3% saccharose and 0.3% gelrite. After 3-4 weeks at 28°C, seedlings were transferred in a Yoshida solution (Yoshida et al. 1976) and maintained at 25/19°C day/night with a 11 h day length. After 4 weeks plants were potted and grown in hothouse until the cycle end.

EXAMPLE 3:

Purification of lactoferrin protein from different tissues of the plant and assessment of molecular weight.

Extraction of all the proteins of tobacco seed was performed grinding the seeds in liquid nitrogen in

presence of an extraction buffer (0.5 M saccharose, 0.1% ascorbic acid, 0.1% Cys-HCl, 0.01 M Tris-HCl, 0.05M EDTA pH 8).

Then the solution was centrifuged for 30 minutes at 14.000 rpm at 4°C and the supernatant was kept with the soluble proteins.

Then the solution was filtered with filters of 0.2 µm porosity, and the lactoferrin partially purified by removing proteins of a molecular weight lower than 30 KDa by centrifugation in Centricon 30 column (Amicon).

The lactoferrin was further purified by HPLC chromatography on Resource Q column (Pharmacia) at a weak cationic exchange, with elution in phosphate buffer pH 7 and NaCl gradient 20-100%. The peak corresponding to lactoferrin eluted at 0.7 M NaCl.

The fractions of the elution range were reunited and filtered in Centricon 30 to remove salt.

For the lactoferrin extraction from tobacco leaves, up to the centrifugation step we proceeded as in the case of extraction from seed, then the supernatant was additioned with 60% $(NH_4)_2SO_4$ and left shaking in ice for 60 min.

Then the solution was centrifuged at 14.000 rpm for 15 minutes at 4°C , the pellet recovered and then suspended again in phosphate buffer pH 6.8.

For the assessment of molecular weight in SDS-PAGE, the colorant (SDS loading buffer) was additioned to the lactoferrin sample (20µl) and the samples were loaded onto 8% polyacrilamide minigels. Running conditions were: initially 10mA, and 20 mA for the entire run, in Tris-glycine 1x buffer. Then the gel was stained by Silver staining technique and the molecular weight assessed referring to molecular weight standards.

EXAMPLE 4:

Western analysis of the lactoferrin protein produced in plant and deglycosilation thereof.

Lactoferrin purified from seed according to example

2, after electrophoretic separation on acrylamide gel was transferred by electroblotting (buffer 25mM Tris, 192 mM glycine, 20% methanol, 45 V at 4°C) to a nitrocellulose membrane (BA85 Schleicher and Schull).

The membrane with the immobilized protein was shaken for 60 min in TBS-T 5% Skin milk solution and then, after some washings, with the same solution containing the primary antibody in a 1:2500 ratio.

After reaction with primary antibody the membrane was washed and placed in contact with the secondary antibody (Anti-Rabbit peroxidase conjugate), always in TBS-T Skin milk solution, in a 1:12.000 ratio.

After reaction with secondary antibody the membrane was washed several times and placed in contact with Amersham's chemiluminescence kit ECL.

The membrane was then exposed in contact with a photoplate (Hyperfilm MP, Amersham) in darkroom for variable lengths of time.

Deglycosilation with N-glycosidase F enzyme (Boehringer Man.) was carried out using 10 µl in volume of glycopeptide (10 µg) denatured in 0.1% SDS brought to boiling point for 2 min.

To this solution 90 µl of buffer (20 mM phosphate buffer pH 7.2, 50 mM EDTA pH 8, 10 mM sodium azide, 0.5& NP40, 1% β-mercaptoethanol) were additioned and it was brought to boiling point again for 2 min, then cooled at 37°C.

To the resulting 100 µl 1 U of N glycosidase F was additioned and let incubate at 37°C for 18 hours. Then the reaction product was analyzed on SDS-PAGE gel and the lactoferrin protein detected by Western technique.

GLOSSARY

The term "recombinant polynucleotide", as it is used here to characterize a polynucleotide useful in the production of lactoferrin, relates to a polynucleotide of genomic origin, cDNA, semi-synthetic or synthetic, that, by virtue of its origin or manipulation: 1) is not

associated to a portion or to the totality of the polynucleotide to which it is associated in nature, and/or 2) is linked to a polynucleotide differing from that to which it is associated in nature, or that 3) does not exist in nature.

The term "polynucleotide", as it is used here, relates to a polymeric form of nucleotides of any length, ribonucleotides as well as deoxyribonucleotides. This term exclusively refers to the molecule primary structure. Hence, the term includes single and double stranded DNA as well as single and double stranded RNA. It also includes modified forms of the polynucleotide, e.g. by methylation, phosphorilation or *"capping"*, and non modified forms.

An «expression cassette for plants» relates to a recombinant polynucleotidic sequence obtained by linking together operatively various elements constituted by the polynucleotidic sequences that determine the in plant expression of a character and that are easily transferable as discrete constructs, from a vector to another by enzymatic restriction.

A "vector" is a replicon to which another polynucleotidic fragment is added, in order to effect the replication and/or expression of the fragment itself.

A "replicon" is any genetic element, for instance a plasmid, a chromosome, a virus, that behaves as an autonomous polynucleotidic replication unit inside a cell; therefore it can replicate autonomously.

"regulation sequence" refers to polynucleotidic sequences that are needed to effect the expression and/or the secretion of coding sequences to which they are bound. The nature of these regulation sequences differs depending on the host; in prokaryotes those regulation sequences usually include promoter, binding site of ribosomes and terminators; in eukaryotes these regulation sequences usually include promoters, terminators and, in some cases, enhancers. In addition, in prokaryotes as well as in eukaryotes, leader sequences control the host cell

secretion of the expressed polypeptide. The term "regulation sequences" includes, at least, all components whose presence is required for expression, and may also include additional components whose presence is advantageous, for instance leader sequences.

A «leader» sequence is a polynucleotidic fragment, usually short, encoding a transport signal of the protein fused thereto and leading the protein transfer into specific cellular compartments. If the transfer takes place through the endoplasmic reticulum the protein undergoes specific posttranscriptional modifications.

"Operatively linked" relates to a juxtaposition wherein the above described components are in a relation enabling them to function in the expected way. A regulation sequence «operatively linked» to a coding sequence is linked in such a way that the coding sequence expression takes place in conditions that are compatible with the regulation sequences.

AN *open reading frame, ORF* is a polynucleotidic sequence region encoding a polypeptide; this region can represent a portion of coding sequence or a complete coding sequence.

A "coding sequence" is a polynucleotidic sequence that is transcripted in the mRNA and/or translated in the polypeptide when placed under control of appropriate regulation sequences. The ends of the coding sequence are determined by a translation start codon at 5' and by a translation stop codon at 3'. A coding sequence can include, without being limited to, mRNA, cDNA, and recombinant polynucleotidic sequences.

"Recombinant host cells", "host cells", "cells", "cell lines", "cell cultures" and other terms indicating microorganisms or cell lines of superior eukaryotes, cultivated as unicellular entities, are used here in an interchangeable way. They relate to cells that can be, or have been, used as hosts for recombinant vectors or other transfer polynucleotides, including the progeny of the

cell that was originally transformed. It is implicit that, due to random or deliberate mutations, the progeny of a single parental cell need not necessarily be identical to the parental cell from a morphological and a genetic point of view. Progenies of the parental cell that are sufficiently similar to the ancestor cell and can be characterized for their salient capacity, as e.g., the presence of a nucleotidic sequence encoding the peptide of interest, are included in the progeny understood according to this definition and fall within the same terms.

For «cell aggregation» a group of cells that are not structured in an organized tissue, but result from an undifferentiated proliferation of cells maintained in particular conditions of hormonal concentration.

"Transformation", as it is used here, refers to the insertion of a exogenous polynucleotide in a host cell, regardless of the method used for the insertion itself, e.g. direct acquisition, Agrobacterium infection, sexual reproduction. The exogenous polynucleotide can be maintained as a non integrated vector, for example a plasmid or, alternatively, it can integrate in the host genome.

As it is used here, the term "polypeptide" relates to the amino acidic product of a sequence encoded inside a genome and does not relate to the specific length of the product: accordingly, peptides, oligopeptides and proteins are included in the definition «polypeptide» This term does not relate to the post-expressional modifications of the peptide, as e.g. glycosilation, acetylation, phosphorilation, sialilation and the like.

A "wild type polypeptide", has an amino acidic sequence identical to the one encoded in the genome of the organism source of the coding sequence.

"Native lactoferrin " and analogous terms relate to the lactoferrin isolated from the source in which it is usually produced in nature by a genome existing in nature.

A "non-native polypeptide " refers to a polypeptide

that is produced in a host differing from the one wherein it is produced in nature.

REFERENCES

- Anderson B.F., Baker H.M., Norris G.E., Rice D.W., Baker E.N. 1989. J. Mol. Biol. 209:711-734.

- Baeuerle P.A. 1995. Nature 373:661-662.

- Bezault J.A., Bhimani R., Wiprovnick J., Furmanski P. 1994. Cancer Res. Baltimore 54:2310-2312.

- Boesman Finkelstein M., Sciortino C.V., Finkelstein R.A., Spik G., Montreuil J., Chrichton R.R., Mazurier J. 1985. Proc. 7th Int. Conf. Prot. Iron Metab. 251-260.

- Bray E., Naito S., Pan N., Anderson E. Dubé P.H., Beachy R.N. 1987. Planta 172:364-370.

- Bustos M.M., Begum D., Kalkan F.A. Battraw M.J., Hall T.C. 1991. EMBO J. 10:1469-1479.

- Chamberland S., Daigle N., Bernier F. 1992. Plant Mol. Biol. 19:937-949.

- Fontes E.P.B., Silva C.J., Carolino S.M.B., Figueiredo J.E.F., Batista D.P.O. 1996. Braz. J. Genetics 19:305-312.

- Fraley R., Schell J. (eds.) 1991. Curr. Opinion Biotechnol, 2:145-210.

- Fujiwara T., Beachy R.N. 1994. Plant Mol. Biol. 24:261-272.

- Galili G., Altschuler Y., Levanony H. 1993. Trends in Cell Biol. 3:437-442.

- Gelvin S.B., Schilperoort R.A. 1995. Plant Molecular Biology Manual. Kluwer Acad. Pub., London.

- Glick B.R., Pasternak J.J. 1994. Molecular Biotechnology. ASM Press, Washington, p. 113.

- Grover M., Giouzeppos O., Schnagl R.D., May J.T. 1997. Acta Paediatr. 86:315-316.

- Hambraeus L., Lonnerdal B. 1993. Indigenous Antimicrobial Agents in Milk-Recent Developments. Pp.97-107.

- Harada J.J., Barker S.J., Goldberg R.B. The Plant Cell, 1:415-425.

- He J., Fumanski P. 1995. Nature 373:721-724.

- Hirano H., Kagawa H., Okubo K. 1989. 31:731-735.

- Ilgoutz S.C., Knittel N., Min Lin J., Sterle S., Gayler K.R. 1997. Plant Mol. Biol. 34:613-627.

- Innis M.A., Gelfand D.H., Sninsky J.J., White T.J. 1990. PCR Protocols: a guide to methods and applications. Academic Press, New York.

- Kagawa H., Yamauchi F., Hirano H. 1987. FEBS Lett. 226:146-149.

- Katagiri F., Lam E., Chua N.H. 1989. Nature 340:727-730.

- Kermode A.R. 1996. Critical Rev. In Plant Sciences, 15:285-423.

- Komatsu S., Hirano H. 1991. FEBS Lett. 294:210-212.

- Lessard P.A., Allen R.D., Bernier F., Crispino J.D., Fujiwara T., Beachy R.N. 1991. Plant Mol. Biol. 16:397-413.

- Lonnerdal B., Iyer S. 1995. Ann. Rev. Nutr.15:93-110.

- Mann D.M., Romm E., Migliorini M. 1995. J. Biol. Chem. 269:23661-23667.

- Marchetti M., Longhi C., Conte M.P., Pisani S., Valenti P., Seganti L. 1996. Antiviral Res. 29:221-231.

- Miehlke S., Reddy R., Osato M.S., Ward P.P., Conneely O.M., Graham D.Y. 1996. J. Clin. Microbiol. 34:2593-2594.

- Mitra A., Zhang Z. 1994. Plant Physiol. 106:977-981.

- Naito S., Dubé P.H., Beachy R.N. 1988. Plant Mol. Biol. 11:109-123.

- Nakajima M., Shinoda I., Samejima Y., Miyauchi H., Fukuwatari Y., Hayasawa H. 1997. J. Cell Physiol. 170:101-105.

- Nishizawa N.K., Mori S., Watanabe Y., Hirano H. 1994. Plant Cell Physiol. 35:1079-1085.

- Nuijens J.H., van Berkel P.H., Geerts M.E., Hartevelt P.P., de Boer H.A., van Veen H.A. 1997. J.

Biol. Chem. 272:8802-8807.

- Oguchi S., Waker W.A., Sanderson I.R. 1995. Biol. Of the Neonate, 67:330-339.

- Ohira K., Ojima K., Fujiwara A. 1973. Studies on the nutrition of rice cell culture.1. A simple defined medium for rapid growth in suspension culture. Plant Cell Physiol. 14:1113-1121.

- Okita T.W. 1996. Ann. Rev. Plant Physiol. Plant Mol. Biol. 47:327-350.

- Owen M.R.L., Pen J. 1996. Transgenic plants: A production system for industrial and pharmaceutical proteins. J. Wiley e Sons, New York.

- Paule-Eugene N., Dugas B., Kolb J.P., Damais C., Braquet P., Paubert-Braquet M., Rialland J.P. 1993. Comp. Rendus Acad. Sci., Series 3, Sci. De la Vie, 316:113-119.

- Penco S., Pastorino S., Bianchi-Scarra G., Garre C. 1995. J. Biol. Chem. 270:12263-12268.

Perez-Grau L., Goldberg R.B. 1989. The Plant Cell, 1:1095-1109.

- Petschow B.W., Talbott R.D. 1991. Pediatric Res. 29:208-213.

- Riggs C.D., Voelker T.A., Chrispeels M.J. 1989. Plant Cell, 1:609-621.

- Salmon V., Legrand D., Georges B., Slomianny M.C., Coddeville B., Spik G. 1997. Protein Expr. Purif. 9:203-210.

- Samaranayake Y.H. et al. 1997. APIMIS, 105:875-883.

- Shewry P.R., Napier J.A., Tatham A.S. 1995. The Plant Cell, 7:945-956.

- Soukka T., Lumikari M., Tenovuo J. 1991. Microbial Ecol. In Health and Dis. 4:259-264.

- Steiner T.S. et al. 1997. Clin. Diagn. Lab. Imm. 4:719-722.

- Stowell K.M., Rado T.A., Funk W.D., Tweedie J.W. 1991. Biochem. J. 276:349-355.

- Superti F., Ammendolia M.G., Valenti P., Seganti

L. 1997. Med. Microb. Imm. 186:83-91.

- Swart P.J., Kuipers M.E., Smit C., Pawels R., De Bethune M.P., De Clercq E., Meijer D.K.F., Huisman J.G. 1996. AIDS Res, and Human Retroviruses, 12:769-775.

- Teraguchi S., Ozawa K., Yasuda S., Shin K., Fukuwatari Y., Shimamura S. 1994. Biosc. Biotech. And Biochem. 58:482-487.

- Teraguchi S., Shin K., Ozawa K., Nakamura S., Fukuwatari Y., Tsuyuki S., Namihara H., Shimamura S. 1995. Appl. Env. Microb. 61:501-506.

- Tomita M. 1993. Ind. Antimicr. Agents of Milk-Recent Develop. pp.7-12.

- Van Berkel P.H.C., Geerts M.E.J., van Veen H.A., Mericskay M., De Boer H.A., Nuijens J.H. 1997. Biochem. J. 328:145-151.

- Yoshida S., Forno D.A., Cook J.H., Gomez K.A. (eds.) 1976. Routine procedures for growing rice plants in culture solution. In: Laboratory manual for physiological studies of rice. IRRI. Los Banos pp. 61-66

- Ward P., Lo J.Y., Duke M., May G.S., Headon D.R., Conneely O.M. 1992. BioTechn. 10:784-789.

- Ward P., Piddington C.S., Cunningham G.A., Zhou X., Wyatt R.D., Conneely O.M. 1995. BioTechn. 13:498-503.

- Ward P., Zhou X., Conneely O.M. 1996. J. Biol. Chem. 271:12790-12794.

- Watanabe Y., Barbashov S.F., Komatsu S., Hemmings A.M., Miyagi M., Tsunasawa S., Hirano H. 1994. Eur. J. Biochem. 224:167-172.

- Watanabe Y., Hirano H. 1994. Plant Physiol. 105:1019-1020.

- Watson J.D., Gilman M., Witkowski J., Zoller M. 1992. Recombinant DNA. W.H. Freeman and Co. New York, p. 213.

- Wobus U., Borisjuk L., Panitz R., Manteuffel R., Baumlein H., Wohlfahrt T., Heim U., Weber H., Misera S., Weschke W. 1995. J. Plant Physiol. 145:592-599.

CLAIMS

1. A polynucleotide coding for human lactoferrin, characterized in that it has a sequence totally or partially corresponding to the sequence reported as SEQ ID NO:1, and in that said sequence is optimized for the in plant expression.

2. The polynucleotide according to claim 1, said polynucleotide having, fused at the 5'-terminus a sequence selected from the group comprising the sequences represented as SEQ ID NO: 13 and NO: 14.

3. Human lactoferrin, characterized in that it is obtained from the expression of a polynucleotide according to claim 1 or 2.

4. A recombinant DNA vector characterized in that it comprises at least one sequence coding for a gene of interest operatively linked to regulation elements allowing the tissue specific expression thereof.

5. The vector according to claim 4, said regulation elements consisting of a plant expression cassette allowing the tissue specific expression of said gene of interest.

6. The vector according to claim 5, wherein said expression cassette for plants consists of the regulation elements of the gene coding for the protein basic globulin 7 S.

7. The vector according to claim 6, wherein the promoter sequence is SEQ ID NO:21

8. The vector according to claim 5, wherein said expression cassette for plants consists of the regulation elements of the gene coding for for protein β-conglycinine.

9. The vector according to claim 8, wherein the promoter sequence is SEQ ID NO:22.

10. The vector according to any one of the claims 4 to 9, wherein the sequence of the gene of interest is operatively linked to a leader sequence.

11. The vector according to claim 10, said leader

sequence being fused to the sequence coding for the gene of interest.

12. The vector according to claim 10 or 11, said sequence leader being selected from the group comprising the sequences according to claim 2.

13. The vector according to any one of the claims 4 to 12, wherein said gene of interest is the human lactoferrin gene.

14. The recombinant DNA vector according to claim 13, said sequence being the sequence according to claim 1.

15. The vector according to any one of the claims 4 to 14, said vector being a plasmid.

16. The vector according to claim 15, said plasmid being selected from the group comprising pUC, pGEM and pBI.

17. The transformation process of plant cells characterized in that said transformation is performed with a vector according to any one of the claims 4 to 16.

18. Transgenic plant cells characterized in that they can be obtained by tranforming of wild type plant cells with a vector according to any one of the claims 4 to 16.

19. Transgenic plant cells characterized in that they contain a gene of interest operatively linked in an expression cassette enabling the tissue specific expression of said gene.

20. The transgenic plant cells according to claim 19, said gene of interest being the one coding for human lactoferrin.

21. The transgenic plant cells according to claim 20, said gene coding for human lactoferrin having a sequence corresponding to the one according to claim 1.

23. The transgenic plant cells according to any one of the claims 19 to 21, wherein said expression cassette includes the regulation region of the gene coding for the 7S basic globulin.

24. The transgenic plant cells according to any one of the claims 19 to 21, wherein said expression cassette includes the control region of the gene coding for the β-conglycinine.

25. Cellular aggregations, characterized in that they can be obtained from cells according to any one of the claims 18 to 24.

26. The cellular aggregations according to claim 25, said aggregations being calluses capable of regenerating transgenic plants.

27. Transgenic plants, characterized in that they can be obtained from the cells according to any one of the claims 18 to 24, by conventional techniques.

28. The transgenic plants characterized in that they contain a gene of interest operatively linked in an expression cassette enabling the tissue specific expression of said gene of interest.

29. The transgenic plants according to claim 28, said gene of interest being the one coding for the human lactoferrin.

30. The transgenic plants according to claim 29, said gene coding for human lactoferrin having a sequence corresponding to the sequence of which at claim 1.

31. The transgenic plants according to any one of the claims 27 a 30, said plants being selected from the group comprising solanaceae, cereals, leguminosae, fruit bearing plants and horticultural plants.

32. The transgenic plant according to claim 31, said plant being soya.

33. The transgenic plant according to claim 31, said plant being tobacco.

34. The transgenic plant according to claim 31, said plant being rice.

35. The transgenic plants according to any one of the claims 27 to 34, said gene being specifically expressed in the storage tissues.

36. The transgenic plants according to claim 35,

said storage tissues being those of the fruit.

37. Use of the transgenic plants according to any one of the claims 27 to 36, as *nutriceuticals*.

38. Production processes of functional foods containing proteins produced by plants, characterized in that the plants according to any one of the claims 27 to 36, are used.

39. The production processes of functional foods according to claim 38, said processes being finalized to the production of vegetal milks, fruit juices, fruit and/or vegetable homogeneized foods.

40. The production processes of vegetal milk, starting from proteins in a native and/or concentrated form, characterized in that proteins yielded from transgenic plants according to any one of the claims 27 to 36 are used.

41. The production process of human lactoferrin, characterized in that transgenic plants according to any one of the claims 27 to 36 are used.

42. Human lactoferrin characterized in that it is obtained from transgenic plants according to any one of the claims 27 to 36.

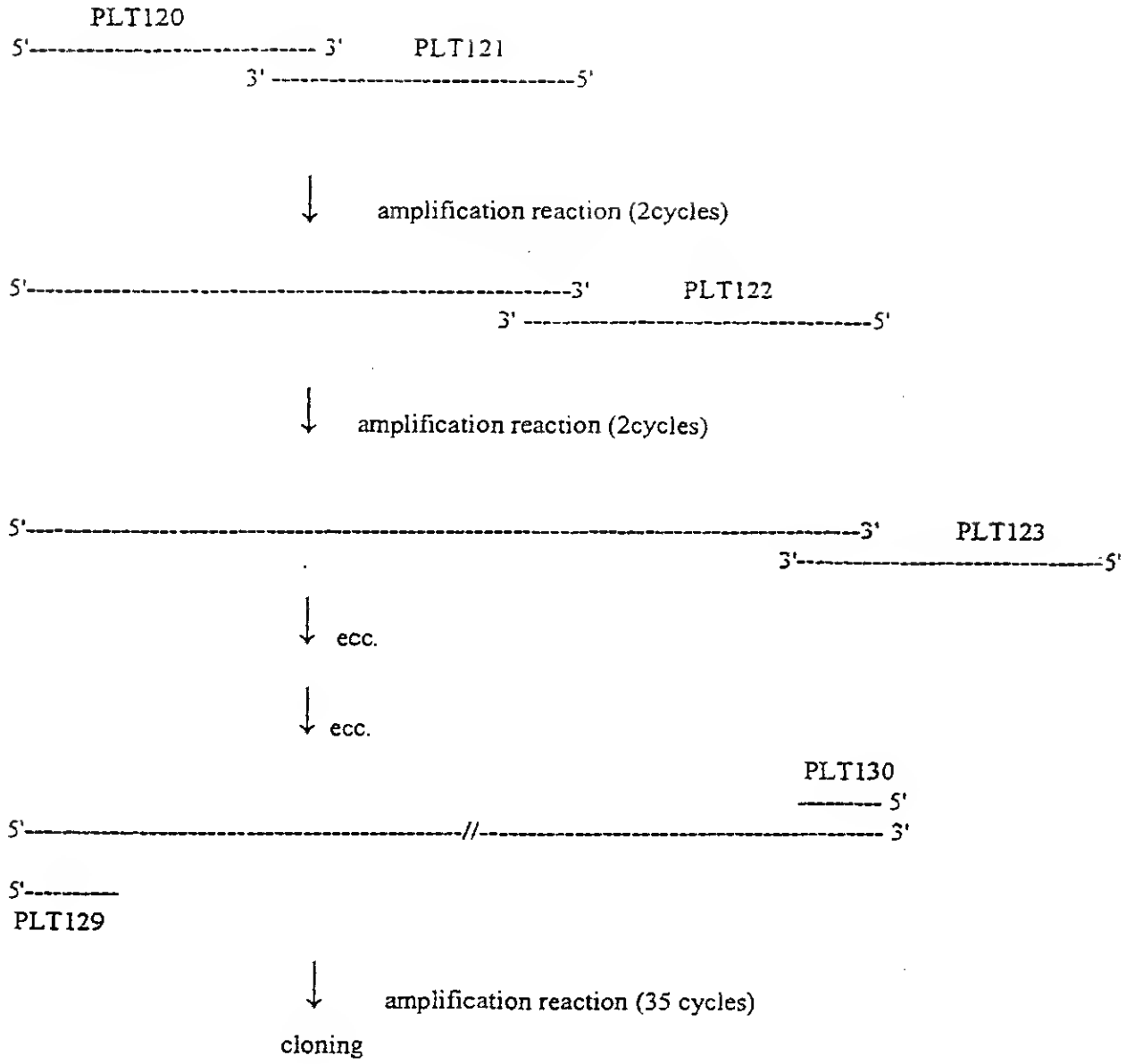
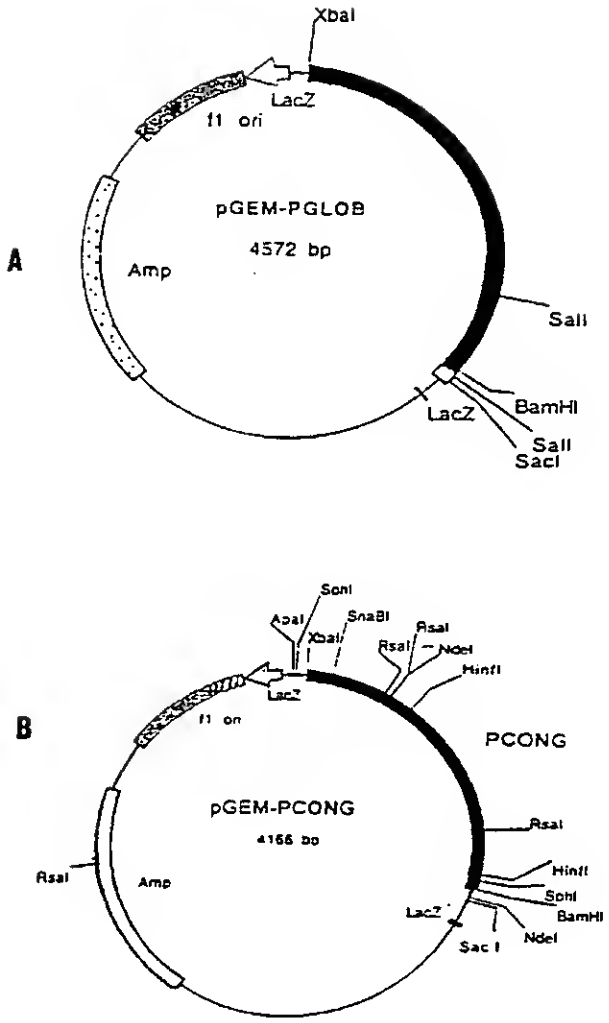

PLT120
5'---------- 3'
PLT121
3' ----------5'
amplification reaction (2cycles)
5'----------3'
PLT122
3' ----------5'
amplification reaction (2cycles)
5'----------3'
PLT123
3'----------5'
ecc.
ecc.
PLT130
5'
5'----------//---------- 3'
5'
PLT129
amplification reaction (35 cycles)
cloning

FIG.1


A
XbaI
LacZ
f1 ori
pGEM-PGLOB
4572 bp
Amp
SalI
LacZ
BamHI
SalI
SacI
B
SohI
ApaI
SnaBI
XbaI
RsaI
RsaI
NdeI
HinfI
LacZ
f1 on
PCONG
pGEM-PCONG
4166 bp
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Amp
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HinfI
SohI
BamHI
NdeI
LacZ
Sac I

FIG.2

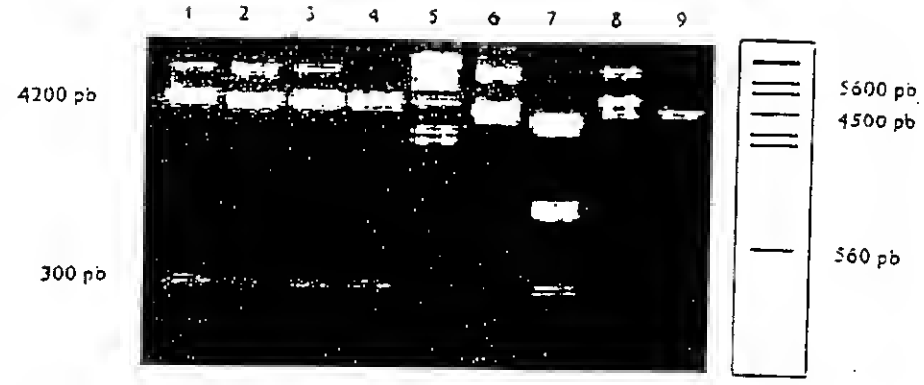



FIG.3

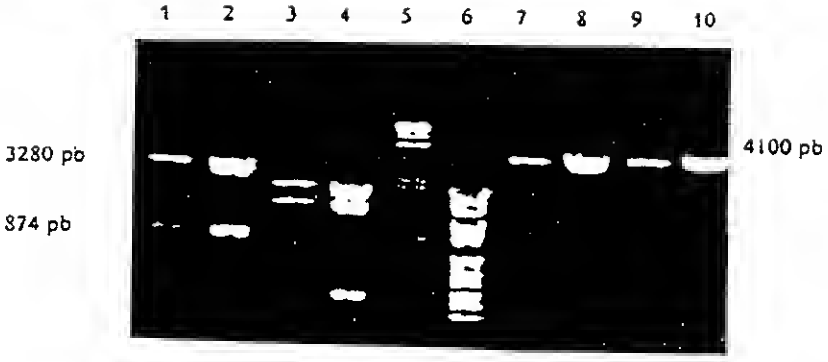



FIG.4

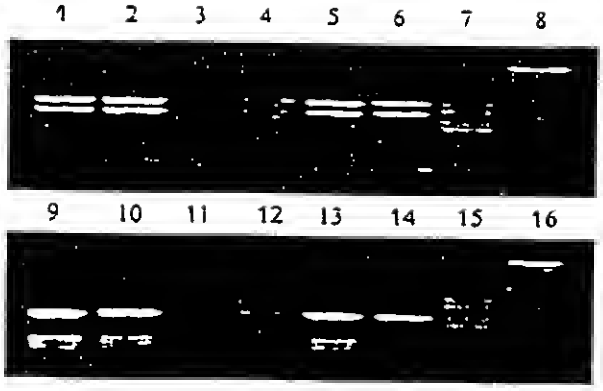



FIG.5

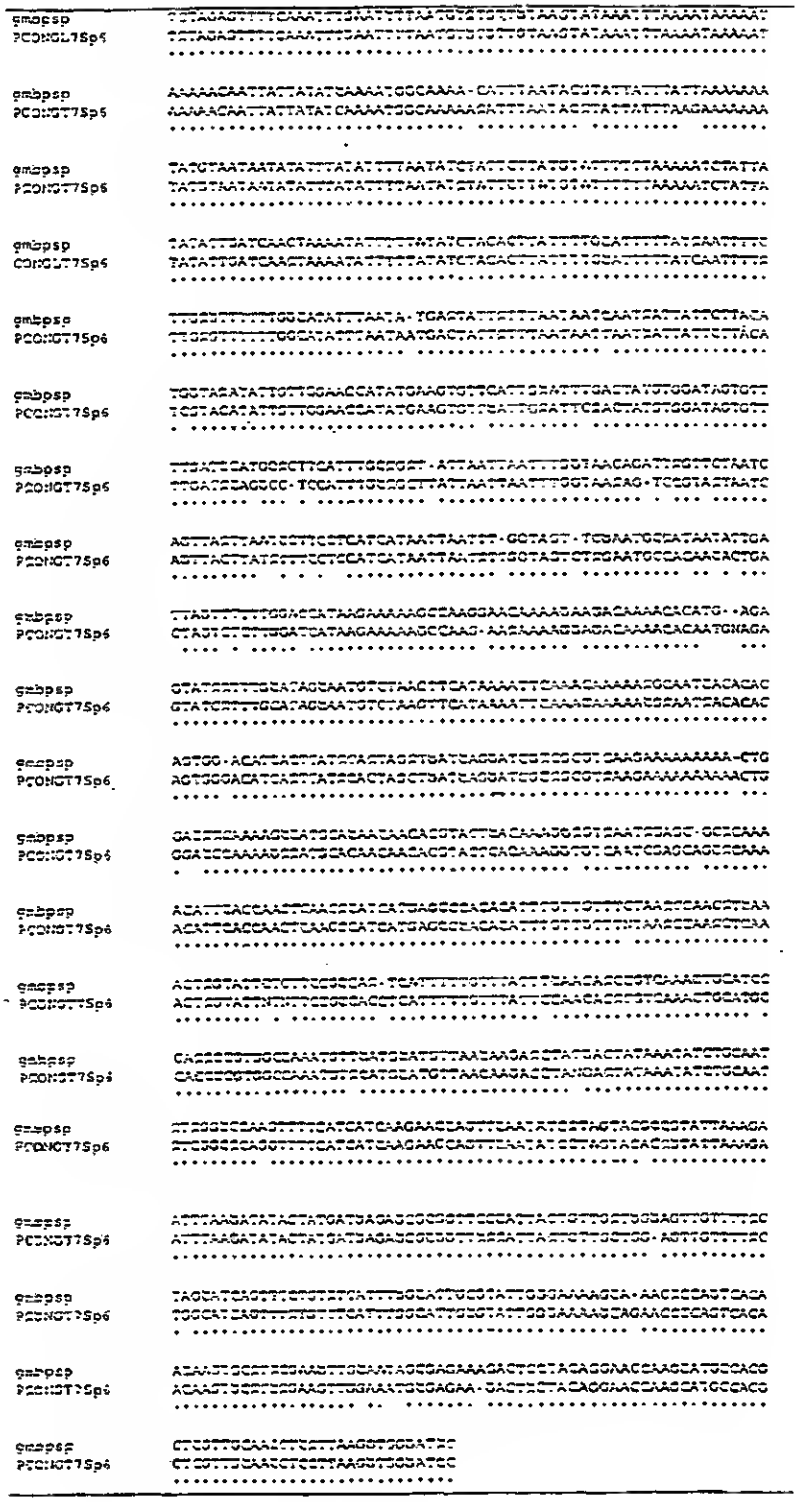
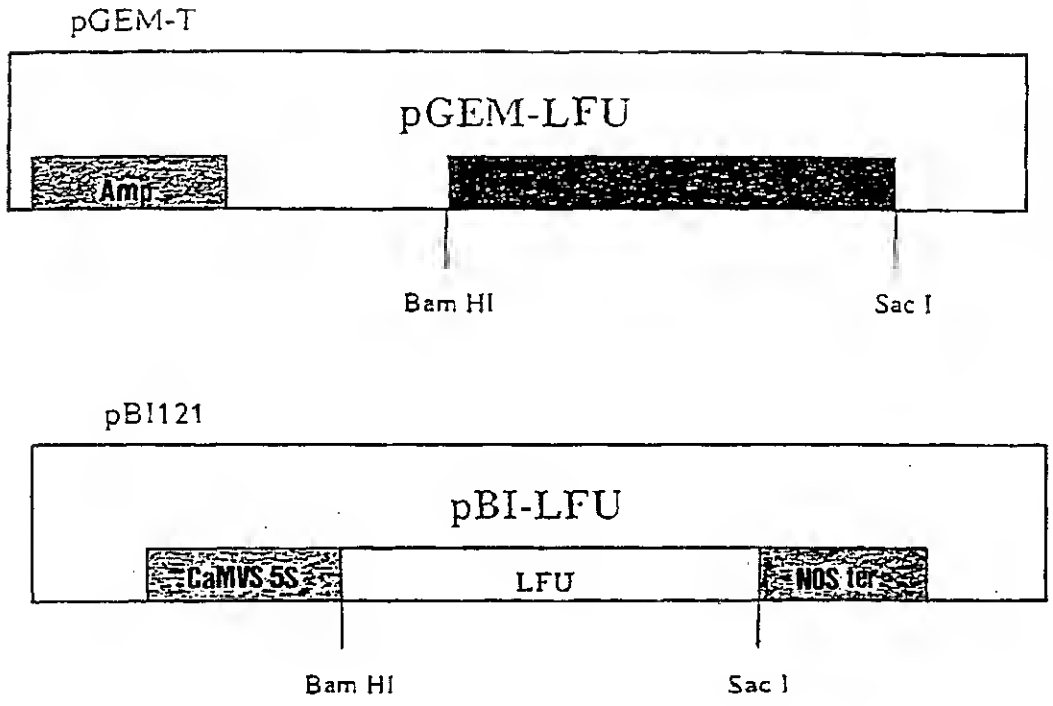


FIG.6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pGEM-LFU
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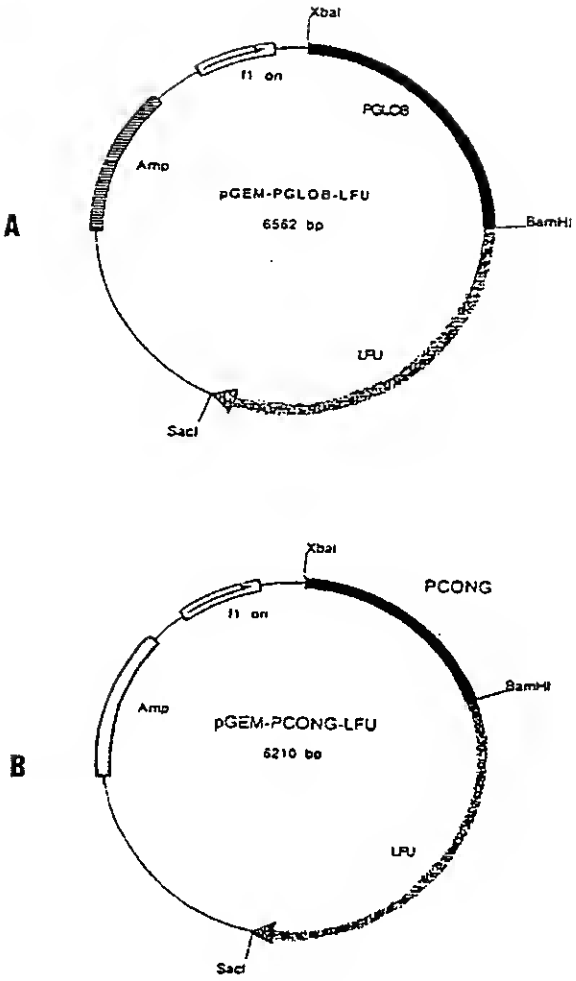
Bam HI
Sac I
pBI121
pBI-LFU
CaMVS 5S
LFU
NOS ter
Bam HI
Sac I

FIG.7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PGLOB
Amp
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BamHI
LFU
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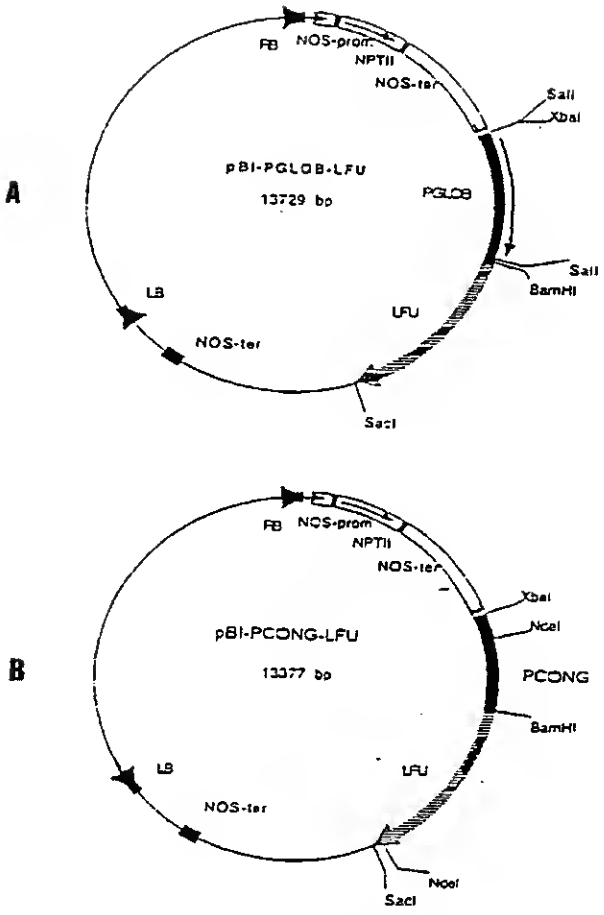
LFU
SacI

FIG.8


A
RB
NOS-prom
NPTII
NOS-ter
SalI
XbaI
pBI-PGLOB-LFU
13729 bp
PGLOB
SalI
BamHI
LB
LFU
NOS-ter
SacI
B
RB
NOS-prom
NPTII
NOS-ter
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PCONG
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LB
LFU
NOS-ter
NcoI
SacI

FIG.9

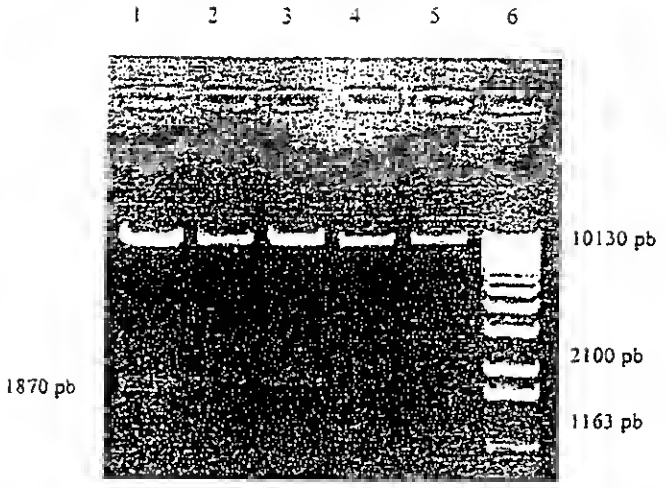
1
2
3
4
5
6
10130 pb
2100 pb
1870 pb
1163 pb

FIG. 10

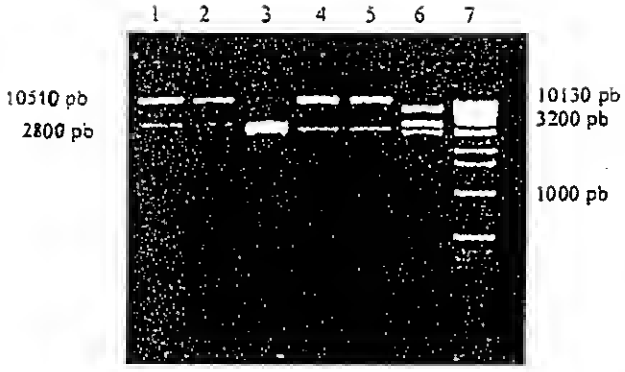
1
2
3
4
5
6
7
10510 pb
2800 pb
10130 pb
3200 pb
1000 pb

FIG. 11

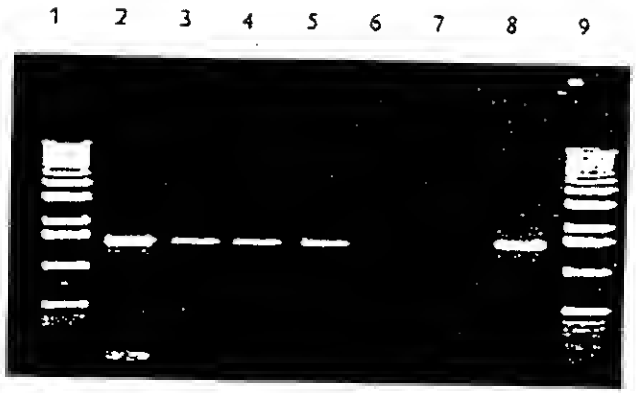


FIG.12

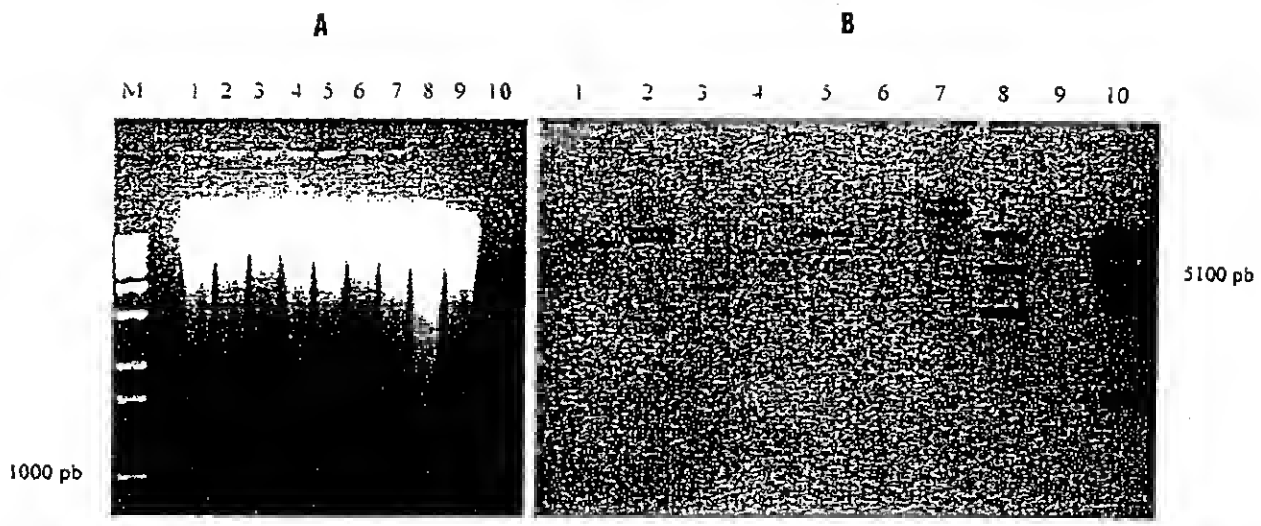


FIG.13

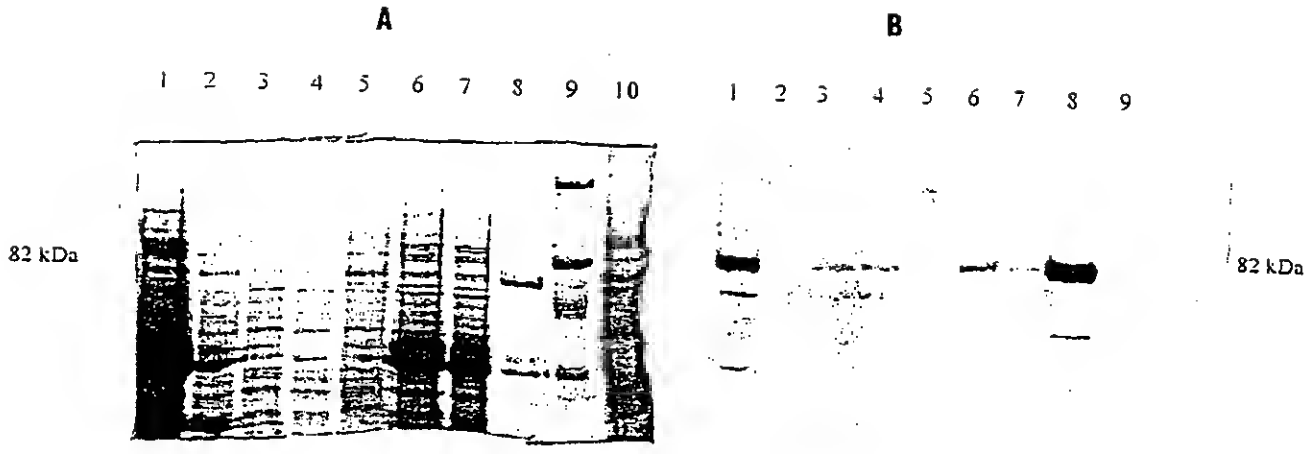


FIG.14

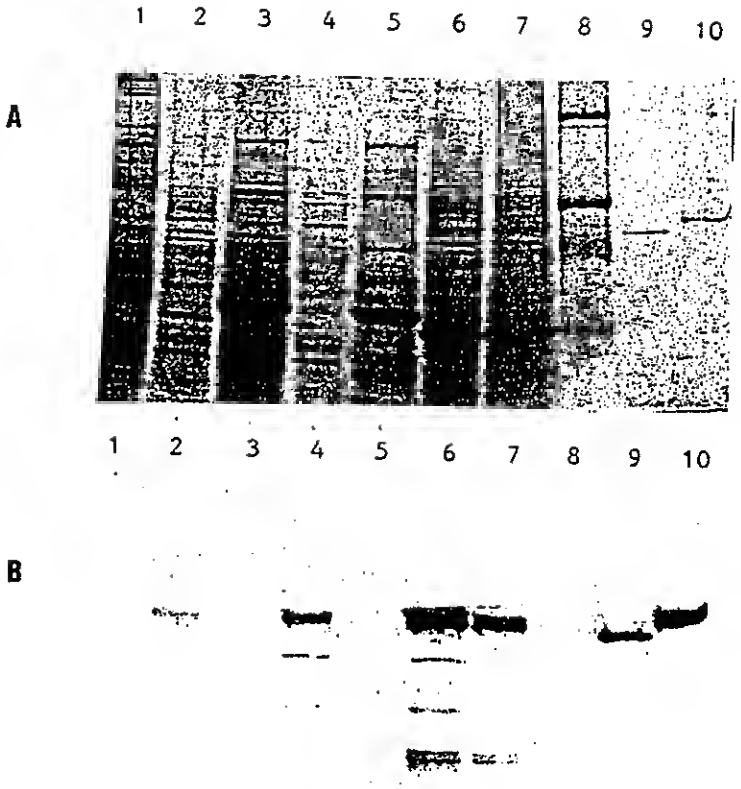


FIG. 15

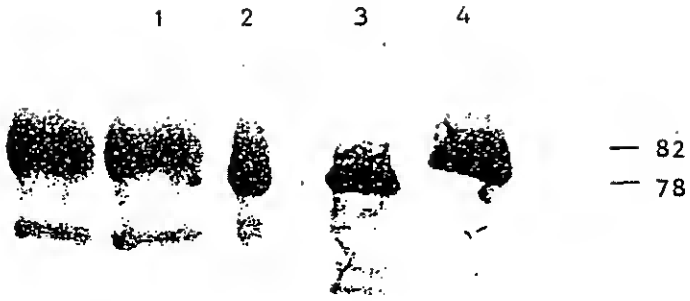


FIG. 16

SEQUENCE LISTING

GENERAL INFORMATION:

(i) APPLICANT: PLANTECHNO S.R.L.

(ii) TITLE OF INVENTION:

SYNTHETIC POLYNUCLEOTIDE ENCODING HUMAN LACTOFERRIN, VECTORS, CELLS AND TRANSGENIC PLANTS CONTAINING IT

(iii) NUMBER OF SEQUENCES: 22

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Società Italiana Brevetti

(B) STREET: Piazza di Pietra, 39

(C) CITY: Roma

(D) COUNTRY: Italy

(E) POSTAL CODE: I-00186

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE:

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS Rev. 5.0

(D) SOFTWARE: Microsoft Word 6.0

(viii) ATTORNEY INFORMATION

(A) NAME: LEONE, Mario (Eng.)

(B) REFERENCE: PC-EBR

(ix) TELECOMMUNICATION INFORMATION

(A) TELEPHONE: 06/695441

(B) TELEFAX: 06/69544830

(C) TELEX: 612287 ROPAT

(1) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 2079 bp

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURES

(A) NAME: LFUSYN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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E   A   T   K   C F   Q   W   Q   R   N   M   R   K   V

CGT GGA CCT CCT GTA TCT TGC ATA AAG AGA GAT TCA CCC ATC CAG 135
R   G   P   P   V   S   C   I   K   R   D   S   P   I Q

TGT ATC CAG GCA ATT GCG GAA AAC AGA GCT GAT GCT GTG ACT CTT 180
C   I   Q   A   I   A   E N   R   A   D   A   V   T   L

GAT GGT GGT TTC ATA TAC GAG GCA GGA CTT GCC CCA TAC AAA CTG  225
D   G   G   F   I   Y   E A   G   L   A   P   Y   K   L

CGA CCT GTA GCG GCG GAA GTC TAC GGG ACC GAA AGA CAA CCA CGA  270
R   P   V   A   A   E   V   Y G   T   E   R   Q   P   R

ACT CAC TAT TAT GCT GTG GCT GTT GTG AAG AAG GGC GGA TCT TTT  315
T   H   Y   Y   A   V   A   V   V   K   K   G   G   S F

CAG CTG AAC GAA CTT CAA GGT CTG AAG TCA TGC CAC ACA GGA CTT  360
Q   L   N   E   L   Q   G   L   K S   C   H   T   G   L

CGC AGG ACC GCT GGA TGG AAT GTC CCT ATA GGG ACA CTT CGT CCA  405
R   R   T   A   G   W   N   V P   I   G   T   L   R   P
```

```
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GCA AGA TTC TTC TCA GCC TCT TGT GTT CCA GGT GCA GAT AAA GGA  495
A   R   F   F   S   A   S   C   V   P   G   A   D   K   G

CAA TTC CCC AAC CTT TGT CGC CTG TGT GCG GGG ACA GGG GAA AAC  540
Q   F   P   N   L   C   R   L   C   A   G   T   G   E   N

AAA TGT GCA TTC TCA TCC CAG GAA CCG TAC TTC AGC TAC TCT GGT  585
K   C   A   F   S   S   Q   E   P   Y   F   S   Y   S   G

GCC TTT AAG TGT CTT AGA GAC GGT GCT GGA GAT GTT GCT TTT ATT  630
A   F   K   C   L   R   D   G   A   G   D   V   A   F   I

AGA GAG AGC ACA GTG TTT GAG GAT CTT TCA GAC GAG GCT GAA AGG  675
R   E   S   T   V   F   E   D   L   S   D   E   A   E   R

GAC GAG TAT GAG TTA CTC TGC CCA GAC AAC ACT CGT AAG CCA GTT  720
D   E   Y   E   L   L   C   P   D   N   T   R   K   P   V

GAC AAG TTC AAA GAT TGC CAT CTT GCA CGG GTC CCT TCT CAT GCC  765
D   K   F   K   D   C   H   L   A   R   V   P   S   H   A

GTT GTG GCA CGA AGT GTT AAT GGA AAG GAG GAT GCC ATC TGG AAT  810
V   V   A   R   S   V   N   G   K   E   D   A   I   W   N

CTT CTC CGC CAA GCA CAG GAA AAG TTT GGA AAG GAC AAG TCA CCG  855
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TTC AAG GAC TCT GCC ATT GGG TTT TCG AGA GTG CCA CCT AGG ATA  945
F   K   D   S   A   I   G   F   S   R   V   P   P   R   I
```

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AAC TTG AGG AAA AGT GAG GAG GAA GTT GCT GCC CGG CGT GCG CGG  1035
N   L   R   K   S   E   E   E   V   A   A   R   R   A   R

GTC GTT TGG TGT GCG GTG GGA GAG CAA GAG TTG CGC AAG TGT AAC  1080
V   V   W   C   A   V   G   E   Q   E   L   R   K   C   N

CAG TGG AGT GGT TTG AGC GAA GGA TCT GTG ACC TGC TCA TCG GCC  1125
Q   W   S   G   L   S   E   G   S   V   T   C   S   S   A

TCC ACT ACA GAA GAT TGC ATC GCC CTG GTG TTG AAA GGA GAA GCT  1170
S   T   T   E   D   C   I   A   L   V   L   K   G   E   A

GAT GCC ATG AGT TTG GAT GGA GGA TAT GTT TAC ACT GCA GGT AAA  1215
D   A   M   S   L   D   G   G   Y   V   Y   T   A   G   K

TGT GGT TTG GTG CCT GTC CTT GCA GAG AAC TAC AAA TCA CAA CAA  1260
C   G   L   V   P   V   L   A   E   N   Y   K   S   Q   Q

AGC AGT GAC CCT GAT CCT AAC TGT GTG GAT AGA CCT GTG GAA GGA  1305
S   S   D   P   D   P   N   C   V   D   R   P   V   E   G

TAT CTT GCT GTG GCG GTG GTT AGG AGA TCA GAC ACT AGC CTT ACC  1350
Y   L   A   V   A   V   V   R   R   S   D   T   S   L   T

TGG AAC TCT GTG AAA GGC AAG AAG TCC TGC CAC ACC GCC GTG GAC  1395
W   N   S   V   K   G   K   K   S   C   H   T   A   V   D

AGG ACT GCA GGT TGG AAT ATC CCC ATG GGA TTG CTC TTC AAC CAG  1440
R   T   A   G   W   N   I   P   M   G   L   L   F   N   Q

ACG GGC TCC TGC AAA TTT GAT GAA TAT TTC AGT CAA AGC TGT GCC  1485
T   G   S   C   K   F   D   E   Y   F   S   Q   S   C   A

CCT GGT TCT GAC CCA AGA TCT AAT CTC TGT GCT TTG TGT ATT GGA  1530
```

```
P  G   S  D  P  R  S  N   L  C  A  L  C  I   G

GAT GAG CAA GGT GAG AAT AAG TGC GTT CCC AAC AGC AAC GAG AGA  1575
D   E   Q   G E   N   K   C   V   P   N S   N   E   R

TAC TAC GGT TAC ACT GGG GCT TTC CGT TGC TTG GCT GAG AAT GCT  1620
Y   Y   G   Y T   G   A   F   R   C   L A   E   N   A

GGA GAC GTT GCA TTT GTG AAA GAT GTC ACT GTC TTG CAG AAC ACT  1665
G   D   V   A F   V   K   D   V   T V   L   Q   N   T

GAT GGA AAT AAC AAT GAG GCA TGG GCT AAG GAT TTG AAG CTT GCA  1710
D   G   N   N   N   E A   W   A   K   D L   K   L   A

GAC TTT  GCG TTG CTG TGC CTC GAT GGC AAA CGT AAG CCT GTG ACT  1755
D   F   A   L   L   C   L   D G   K   R   K P   V   T

GAA GCT AGA AGC TGC CAT CTT GCC ATG GCC CCG AAT CAT GCT GTG  1800
E   A   R   S C   H   L   A   M   A P   N   H   A   V

GTG TCT CGT ATG GAT AAG GTG GAA CGC TTG AAA CAG GTG TTG CTC  1845
V   S   R   M D   K   V   E   R L   K   Q   V   L   L

CAC CAA CAG GCT AAA TTT GGT AGA AAT GGA TCT GAC TGC CCG GAC  1890
H   Q   Q   A   K   F G R   N   G S   D   C   P   D

AAG TTT TGC TTA TTC CAG TCT GAA ACC AAA AAC CTT TTG TTC AAT  1935
K   F   C   L F   Q   S   E   T K   N   L   L F   N

GAC AAC ACT GAG TGT CTT GCC AGA CTC CAT GGC AAA ACA ACA TAT  1980
D   N   T   E   C L   A   R   L H   G   K   T   T   Y

GAA AAA TAT TTG GGA CCA CAG TAT GTC GCA GGC ATT ACT AAT CTG  2025
E   K   Y   L   G P   Q   Y   V   A   G I   T   N   L

AAA AAG TGC TCA ACC TCC CCA CTC CTA GAA GCC TGT GAA TTC CTA  2070
K   K   C S   T   S   P   L   L   E   A C   E   F   L
```

AGG AAG TAA 2079
R K *

(2) INFORMATION FOR SEQ ID NO: 2:
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 30 bp
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: oligonucleotide
(ix) FEATURES
(A) NAME: PLT46
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GGATCCATGG GCCGTAGGAG AAGGAGTGTT 30

(3) INFORMATION FOR SEQ ID NO: 3:
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 32 bp
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: oligonucleotide
(ix) FEATURES
(A) NAME: PLT 47
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GAGCTCCTTC GGTTTTACTT CCTGAGGAAT TC 32

(4) INFORMATION FOR SEQ ID NO: 4:
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 42 bp
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: oligonucleotide
(ix) FEATURES

(A) NAME: PLT 48

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TCTAGATAAA ATAATCTATA CATTAAAAAA TTTGATTTTA AA 42

(5) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 36 bp

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(ix) FEATURES

(A) NAME: PLT 49

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GGATCCGACT GAGTCGGATA AGAAGAAAAG AAAAGA 36

(6) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 36 bp

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(ix) FEATURES

(A) NAME: PLT 50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TCTAGAGTTT TCAAATTTGA ATTTTAATGT GTGTTG 36

(7) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 36 bp

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: oligonucleotide
(ix) FEATURES
(A) NAME: PLT 51
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GGATCCCACC TTAAGGAGGT TGCAACGAGC GTGGCA 36

(8) INFORMATION FOR SEQ ID NO:8:
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 250 bp
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: oligonucleotide
(ix) FEATURES
(A) NAME: PLT 120
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGC CGT AGG AGA AGG AGT GTT CAA TGG TGC GCA GTA TCA CAA CCA GAG

GCC ACA AAA TGC TTC CAA TGG CAA AGG AAT ATG AGA AAA GTT CGT GGA

CCT CCT GTA TCT TGC ATA AAG AGA GAT TCA CCC ATC CAG TGT ATC CAG

GCA ATT GCG GAA AAC AGA GCT GAT GCT GTG ACT CTT GAT GGT GGT TTC

ATA TAC GAG GCA GGA CTT GCC CCA TAC AAA CTG CGA CCT GTA GCG GCG

GAA GTC TAC G

(9) INFORMATION FOR SEQ ID NO:9:
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 250 bp
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(ix) FEATURES

(A) NAME: PLT 121

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GC ACC TGG AAC ACA AGA GGC TGA GAA GAA TCT TGC CAC AGC TGC CTC

AAT GGG CTC AGG TGG ACC CGT CCA ATT CAA GAA TGG ACG AAG TGT CCC

TAT AGG GAC ATT CCA TCC AGC GGT CCT GCG AAG TCC TGT GTG GCA TGA

CTT CAG ACC TTG AAG TTC GTT CAG CTG AAA AGA TCC GCC CTT CTT CAC

AAC AGC CAC AGC ATA ATA GTG AGT TCG TGG TTG TCT TTC GGT CCC GTA

GAC TTC CGC CG

(10) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 250 bp

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(ix) FEATURES

(A) NAME: PLT 122

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AAC TGG CTT ACG AGT GTT GTC TGG GCA GAG TAA CTC ATA CTC GTC

CCT TTC AGC CTC GTC TGA AAG ATC CTC AAA CAC TGT GCT CTC TCT

AAT AAA AGC AAC ATC TCC AGC ACC GTC TCT AAG ACA CTT AAA GGC

ACC AGA GTA GCT GAA GTA CGG TTC CTG GGA TGA GAA TGC ACA TTT

GTT TTC CCC TGT CCC CGC ACA CAG GCG ACA AAG GTT GGG GAA TTG

TCC TTT ATC TGC ACC TGG AAC ACA A

(11) INFORMATION FOR SEQ ID NO:11:
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 255 bp
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: oligonucleotide
(ix) FEATURES
(A) NAME: PLT 123
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GTA CAA CCC AGA ATC TAT CCT AGG TGG CAC TCT CGA AAA CCC AAT GGC

AGA GTC CTT GAA CAG AAG ATC TTT CTG CCC ACT AGG GGA ACC AAA GAG

CTG GAA TTT CGG TGA CTT GTC CTT TCC AAA CTT TTC CTG TGC TTG GCG

GAG AAG ATT CCA GAT GGC ATC CTC CTT TCC ATT AAC ACT TCG TGC CAC

AAC GGC ATG AGA AGG GAC CCG TGC AAG ATG GCA ATC TTT GAA CTT GTC

AAC TGG CTT ACG AGT

(12) INFORMATION FOR SEQ ID NO:12:
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 251 bp
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: oligonucleotide
(ix) FEATURES
(A) NAME: PLT 124
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TA TCC TCC ATC CAA ACT CAT GGC ATC AGC TTC TCC TTT CAA CAC CAG

GGC GAT GCA ATC TTC TGT AGT GGA GGC CGA TGA GCA GGT CAC AGA TCC

TTC GCT CAA ACC ACT CCA CTG GTT ACA CTT GCG CAA CTC TTG CTC TCC

CAC CGC ACA CCA AAC GAC CCG CGC ACG CCG GGC AGC AAC TTC CTC CTC

ACT TTT CCT CAA GTT CTG AAT TGC AGT AAA GTA TCC GGA GCC AAG GTA

CAA CCC AGA ATC

(13) INFORMATION FOR SEQ ID NO:13:
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 75 bp
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: oligonucleotide
(ix) FEATURES
(D) OTHER INFORMATION: leader sequence of protein 7S basic globulin
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:
ATGGCTTCTA TCCTCCACTA CTTTTTAGCC CTCTCTCTTT CTTGCTCTTT 50
TCTTTTCTTC TTATCCGACT CAGTC 75

(14) INFORMATION FOR SEQ ID NO:14:
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 191 bp
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: oligonucleotide
(ix) FEATURES
(D) OTHER INFORMATION: leader sequence of protein β-conglycinine
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

ATGATGAGAG CGCGGTTCCC ATTACTGTTG CTGGGAGTTG TTTTCCTAGC 50
ATCAGTTTCT GTCTCATTTG GCATTGCGTA TTGGGAAAAG CAGAACCCCA 100
GTCACAACAA GTGCCTCCGA AGTTGCAATA GCGAGAAAGA CTCCTACAGG 150
AACCAAGCAT GCCACGCTCG TTGCAACCTC CTTAAGGTG 189

(15) INFORMATION FOR SEQ ID NO:15:
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 250 bp
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: oligonucleotide
(ix) FEATURES
(A) NAME: PLT 125
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:
GAGCAATCCC ATGGGGATAT TCCAACCTGC AGTCCTGTCC ACGGCGGTGT 50
GGCAGGACTT CTTGCCTTTC ACAGAGTTCC AGGTAAGGCT AGTGTCTGAT 100
CTCCTAACCA CCGCCACAGC AAGATATCCT TCCACAGGTC TATCCACACA 150
GTTAGGATCA GGGTCACTGC TTTGTTGTGA TTTGTAGTTC TCTGCAAGAC 200
AGGCACCAAA CCACATTTAC CTGCAGTGTA AACATATCCT CCATCCAAAC 250

(16) INFORMATION FOR SEQ ID NO:16:
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 254 bp
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: oligonucleotide
(ix) FEATURES
(A) NAME: PLT 126
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:
CCATCAGTGT TCTGCAAGAC AGTGACATCT TTCACAAATG CAACGTCTCC 50
AGCATTCTCA GCCAAGCAAC GGAAAGCCCC AGTGTAACCG TAGTATCTCT 100
CGTTGCTGTT GGGAACGCAC TTATTCTCAC CTTGCTCATC TCCAATACAC 150
AAAGCACAGA GATTAGATCT TGGGTCAGAA CCAGGGGCAC AGCTTTGACT 200
GAAATATTCA TCAAATTTGC AGGAGCCCGT CTGGTTGAAG AGCAAGCCCA 250
TGGG 254

(17) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 229 bp

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(ix) FEATURES

(A) NAME: PLT 127

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GCAGTCAGAT CCATTTCTAC CAAATTTAGC CTGTTGGTGG AGCAACACCT 50

GTTTCAAGCG TTCCACCTTA TCCATACGAG ACACCACAGC ATGATTCGGG 100

GCCATGGCAA GATGGCAGCT TCTAGCTTCA GTCACAGGCT TACGTTTGCC 150

ATCGAGGCAC AGCAACGCAA AGTCTGCAAG CTTCAAATCC TTAGCCCATG 200

CCTCATTGTT ATTTCCATCA GTGTTCTGC 229

(18) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 210 bp

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(ix) FEATURES

(A) NAME: PLT 128

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TTACTTCCTT AGGAATTCAC AGGCTTCTAG GAGTGGGGAG GTTGAGCACT 50

TTTTCAGATT AGTAATGCCT GCGACATACT GTGGTCCCAA ATATTTTTCA 100

TATGTTGTTT TGCCATGGAG TCTGGCAAGA CACTCAGTGT TGTCATTGAA 150

CAAAAGGTTT TTGGTTTCAG ACTGGAATAA GCAAAACTTG TCCGGGCAGT 200

CAGATCCATT 210

(19) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 30 bp

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(ix) FEATURES
(A) NAME: PLT 129
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GGATCCATGG GCCGTAGGAG AAGGAGTGTT 30

(20) INFORMATION FOR SEQ ID NO:20:
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 28 bp
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: oligonucleotide
(ix) FEATURES
(A) NAME: PLT 120
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:
GAGCTCTTAC TTCCTTAGGA ATTCACAG 28

(21) INFORMATION FOR SEQ ID NO:21:
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: bp
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA
(ix) FEATURES
(D) OTHER INFORMATION: sequence of promoter of gene encoding 7S basic globulin
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TAAAATAATC TATACATTAA AAAATTTGAT TTTAAAATTT TAGAAATTCA 50
TGATTTTATT TTTTTTTACC AGAAATCCGT TAATATTGTT AAAATATTAC 100
CAACTAATTT ATAAATTTTA TTTTAAGGCA ATTAAGCATG TTTGATAAAA 150
TATATATATT GTTATAAATA CTTTTCAAAA GTATAAAGTT GATGATGGCG 200
TGGTGGTAGA TTATTTTAGT TCTAGGTTCG AATGCAAGTT GGTTTAGACA 250
TTTAGCCTTA TTCTTTTTTC TAACCAAAAT AAATGTAAAT GGAAAACCTT 300
TAGGAAAAAA AAGAAATCAA AATTGAAAAC ATCATCCGGT GGAGTCGAGA 350
AGCCCACACC CACGTGACCC AACAATATTA AAATAAGAGT TTGCTCTACA 400

GTAAATGCGA TACTTTTTTA TTCAATACTT TTTCCACTTC TAAAATCTTG 450
GAGATTTGCA CCGTTAACTA ATTAAGTGTT ATATCCAACG GTCCTAAAAA 500
AACTTGTGTA CCGTGCCTCA CATTTCAACT TTGCGCACCC TGAAAGCCGT 550
TATGTTTAGG TTAGTGTTTG CAACAGTTGA AGCGCATCAC TCAGGAGGCT 600
ACTTGGTCTT GCTTTTGCGT CTTTTGTTCA ATTTTTCACG TGATTTTGTT 650
GGTGAACACG CGTACTTGAA ACTTATTATA AATTACATAA TTTTATAAGT 700
TTCACTTCTT ATATAATACT CATATAATAT ATAGGGTTTA GAATGCCAAT 750
TTTTAAAAAA AGAATAAAAA AATAAATAGA ATAAAATCGA AAAAATGAAA 800
TGTAAAAAAT TTGAGGGGGA CAAATAAAAT ATGAAAGTCT ATTATTTAAA 850
TTTTCCATTA GAATTCTATT TTCCTTAGTT AATATGAGCT AGCCAGTTGG 900
GAGATACACG AAAATGTCAT GAAACAGTTG CATGTAGGGA AATTAATGTA 950
GTAGAGGGAT AGCAAGACAA AAATCCAAGC CAAGCTAGCT GCTCACGCGA 1000
ACTCGATCCA CACGTCCTTT ACAGAGTTTC AAACGGATGA AATCTGCATG 1050
GCATGCAACT AAAGCATTGT TCTCAGCTGC CAAGTACCCC TCACACTCAC 1100
CAACCCTTTG TTTTTCTCCC CATTGCATGT TAACTCAAGT TTATCCTTTC 1150
TTTGCTTCTG GAAATTTCAC AAGCCTCAAA CACGTCGACG TCCAATCTTG 1200
TGACCAACAC GGCCAAAAGA AAAGAGAATC TCATCCCGTT CACACTTAGC 1250
CACTTAAAGC TAGCCAAACG GTGATCTTTC TCTATATATT GTAGCTCTCT 1300
AACACAACCA ACACTACCAT TATTCAATAT TCAAACCTTG CTCTATACTA 1350
CACACACTAG AAGAATA 1367

(22) INFORMATION FOR SEQ ID NO:22:
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: bp
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA
(ix) FEATURES
(D) OTHER INFORMATION: sequence of promoter of gene coding for β-conglycinine
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:
GTTTTCAAAT TTGAATTTTA ATGTGTGTTG TAAGTATAAA TTTAAAATAA 50
AAATAAAAAC AATTATTATA TCAAAATGGC AAAAACATTT AATACGTATT 100
ATTTATTAAA AAAATATGTA ATAATATATT TATATTTTAA TATCTATTCT 150
TATGTATTTT TTAAAAATCT ATTATATATT GATCAACTAA AATATTTTTA 200
TATCTACACT TATTTTGCAT TTTTATCAAT TTTCTTGCGT TTTTTGGCAT 250

ATTTAATAAT GACTATTCTT TAATAATCAA TCATTATTCT TACATGGTAC 300
ATATTGTTGG AACCATATGA AGTGTTCATT GCATTTGACT ATGTGGATAG 350
TGTTTTGATC CATGCCCTTC ATTTGCCGCT ATTAATTAAT TTGGTAACAG 400
ATTCGTTCTA ATCAGTTACT TAATCCTTCC TCATCATAAT TAATCTGGTA 450
GTTCGAATGC CATAATATTG ATTAGTTTTT TGGACCATAA GAAAAAGCCA 500
AGGAACAAAA GAAGACAAAA CACAATGAGA GTATCCTTTG CATAGCAATG 550
TCTAAGTTCA TAAAATTCAA ACAAAAACGC AATCACACAC AGTGGACATC 600
ACTTATCCAC TAGCTGATCA GGATCGCCGC GTCAAGAAAA AAAAACTGGA 650
CCCCAAAAGC CATGCACAAC AACACGTACT CACAAAGGCG TCAATCGAGC 700
AGCCCAAAAC ATTCACCAAC TCAACCCATC ATGAGCCCAC ACATTTGTTG 750
TTTCTAACCC AACCTCAAAC TCGTATTCTC TTCCGCCACC TCATTTTTGT 800
TTATTTCAAC ACCCGTCAAA CTGCATCCCA CCCCGTGGCC AAATGTTCAT 850
GCATGTTAAC AAGACCTATG ACTATAAATA TCTGCAATCT CGGCCCAAGT 900
TTTCATCATC AAGAACCAGT TCAATATCCT AGTACGCCGT ATTAAAGAAT 950
TTAAGATATA CT 962

# INTERNATIONAL SEARCH REPORT

In. rtional Application No
PCT/IT 99/00226

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C12N15/82 C12N5/10 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | SALMON V. ET AL.: "Production of human lactoferrin in transgenic tobacco plants" PROTEIN EXPRESSION AND PURIFICATION, vol. 13, 1998, pages 127-135, XP000863470 | 1,3, 37-42 |
| Y | see the whole document, esp. discussion | 2,4,5,8, 9,19-21, 24, 28-30, 35,36 |

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 23 December 1999 | 11/01/2000 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax (+31-70) 340-3016 | Kania, T |

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | MITRA A ET AL: "EXPRESSION OF A HUMAN LACTOFERRIN CONA IN TO8ACCO CELLS PRODUCES ANTIBACTERIAL PROTEIN(S)" PLANT PHYSIOLOGY,US,AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, ROCKVILLE, MD, vol. 106, no. 3, 1994, page 977-9B1 XP002048441 ISSN: 0032-0889 the whole document | 3,42 |
| X | WARD P P ET AL: "A SYSTEM FOR PROOUCTION OF COMMERCIAL OUANTITIES OF HUMAN LACTOFERRIN: A BROAD SPECTRUM NATURAL ANTIBIOTIC" BIO/TECHNOLOGY,US,NATURE PUBLISHING CO. NEW YORK, vol. I3, I995, page 498-503 XP002048442 ISSN: 0733-222X the whole document | 3,42 |
| X | ZHANG ET AL: "DEVELOPMENT OF TRANSGENIC PLANTS WITH NON-PLANT ANTIBACTERIAL PROTEIN GENES FOR RESISTANCE TO BACTERIAL PATHOGENS ( LACTOFERRIN, NICOTIANA TABACUM, AGRO8ACTERIUM TUMEFACIENS)" DIALOG DISSERTATION A8STRACTS,XX,XX,1996, XP002048439 abstract | 3,42 |
| X | WO 96 37094 A (KOREA INST SCIENCE TECHNOLOGY ;LIU JANG RYOL (KR); LEE KYUNG KWANG) 28 November 1996 (1996-1I-28) the whole document | 3,42 |
| X | WO 9I I3993 A (UPJOHN CO) I9 September 1991 (I991-09-I9) | 4,5, 8-I1, I5-I9, 24-2B, 3I-36 |
| Y | see the whole document, esp. examples 4 and 9 | 2,4,5,8, 9,I9-21, 24, 2B-30, 35,36 |
| A | WO 98 06860 A (CI8A GEIGY AG ;VERNOOIJ BARNAROUS THEOOORUS M (NL); CHANDLER DANIE) 19 February I998 (I998-02-19) see the whole document, esp. page 4, example 1I | I-42 |

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | WATANABE Y ET AL: "Nucleotide sequence of the basic 7S globulin gene from soybean." PLANT PHYSIOLOGY, (1994 JUL) 105 (3) 1019-20. , XP002125177 the whole document | 6,7,23 |
| P,X | FR 2 762 850 A (BIOCEM) 6 November 1998 (1998-11-06) the whole document | 3-5,10, 11,13, 15-20, 25-29, 31-42 |

# INTER TIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IT 99/00226

| Patent document cited in search report | Publication dat | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 9637094 A | 28-11-1996 | EP 0831688 A | 01-04-1998 |
| | | JP 10506540 T | 30-06-1998 |
| | | US 5914448 A | 22-06-1999 |
| WO 9113993 A | 19-09-1991 | AU 7583691 A | 10-10-1991 |
| | | EP 0519011 A | 23-12-1992 |
| WO 9806860 A | 19-02-1998 | AU 4205297 A | 06-03-1998 |
| | | CN 1228123 A | 08-09-1999 |
| | | EP 0918873 A | 02-06-1999 |
| | | PL 331563 A | 19-07-1999 |
| FR 2762850 A | 06-11-1998 | AU 7659298 A | 27-11-1998 |
| | | WO 9850543 A | 12-11-1998 |